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THE EUROPEAN CORN BORER, PYRAUSTA NUBILALIS (HUBN), AND ITS PRINCIPAL HOST PLANT. V. A CHEMICAL STUDY OF HOST PLANT RESISTANCE[1,2]

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The European corn borer is a major insect pest of corn (*Zea Mays* L.) throughout the corn belt of the United States, causing an annual loss of over 100 million dollars (14). The development of adequate and economical measures of suppressing populations of this insect is a problem which has concerned entomologists for nearly forty years (see Painter (20) for review).

A desirable method of combating the borer, or any phytophagous insect, is through the development and use of resistant or immune plant varieties. Although it has been well established that corn varieties differ in their relative susceptibility to corn borer attack (12, 13, 21, and many others), no agriculturally acceptable corn hybrids have been produced which display more than a slight resistance toward the borer. There are perhaps several reasons for such limited success. The physical and chemical factors contributing to resistance have never been resolved sufficiently to provide the corn geneticists with the information needed for the development of genetic lines displaying defined resistance. Resistance toward borer feeding has been shown to be dependent, at least in part, on the developmental stage of the plant at the time of borer attack, and varietal differences in resistance have not been separated clearly from varietal differences in growth characteristics (8, 11, 15, 19, 25). Elucidation of the factors contributing to the insect resistance of a plant variety is not solely an entomological problem, but is in an area of research calling for the attention of the plant physiologist as well as the entomologist.

The development of a satisfactory purified diet and aseptic culturing technique for European corn borer larvae (9, 10) has made it possible to study the borer's nutritional and feeding requirements (2, 3). An experimental approach to the problem of the adaptation of the borer larva to the corn plant has also been made (1, 4, 5, 6, 11). Evidence has been obtained that inhibitors of borer growth and survival occur in the leaves of young corn plants (1, 11). These substances were found to be present in water extracts of young corn plants of the several varieties tested, and it was also shown that some of the substances which affect borer growth also inhibit the growth of a variety of organisms, including *Penicillium chrysogenum*. As a result of the latter discovery a bioassay method was devised using the fungus as the bioassay organism (11).

The work reported herein was initiated in the fall of 1953 and was directed toward an elucidation of the chemical nature of the growth inhibitors contained in corn plants and their distribution among the tissues of corn plants of different ages and varieties.

MATERIALS AND METHODS

Field and greenhouse grown corn plants of three inbred lines (W 22 RB and W 210 D, resistant; W 204, susceptible) were used in most of the work. The plants were harvested by cutting them off at ground level, and after being grouped or partitioned into suitable samples, they were placed in polyethylene bags, frozen immediately, and stored at – 15° C until needed. For work on isolation of growth inhibitors, whole plants of 20 to 30 inches in extended height were ordinarily used.

In developing a scheme for the isolation of the inhibitors, various fractions could be tested for activity by adding them to diets on which the corn borer larvae were reared. However, because routine assays using the corn borer were difficult and variable, the Penicillium bioassay was adopted and used as described below.

The basal medium for the bioassay consisted of 2 % aqueous agar containing 1 % Bacto-peptone and 6 % honey. Aqueous solutions to be assayed were added to the medium at the expense of the water in the medium. Fifty ml of medium were prepared for each assay and after being autoclaved it was distributed between two sterilized Petri dishes. Control plates were prepared in a similar fashion with basal medium. When the assay plates had cooled, they were innoculated with one ml of a suspension containing 10 to 15 fungus spores per ml, and placed in a controlled temperature room (24 to 25° C). Seventy-two hours after inoculation the diameters of five Penicillium colonies on each plate were measured.

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The activity of the solutions, calculated as percent inhibition of growth, could then be expressed in terms of specific activity per gram fresh or dry weight of corn tissue which the 50-ml volume of assay medium contained. The fresh weight basis appeared to be of more significance in relation to the feeding habits of the corn borers. The inhibition of the fungus gave a linear regression with increasing concentrations of the inhibitors and activity values could be compared directly for quantative purposes. Statistical analyses of several assays revealed that five percent inhibition of growth was significant at the 0.05 confidence level.

Utilizing the Penicillium and corn borer assays, a fractionation scheme was worked out for the partial isolation of one of the inhibitors. It involved the preparation of an aqueous extract of fresh or frozen corn tissue by homogenizing in water, boiling and finally filtering. Norite "A" charcoal was added to the hot filtrate which was then allowed to cool. Concentrated KOH was added to the suspension which was then filtered. The charcoal was slurried in 80 % ethanol and filtered again and the charcoal discarded The ethanol solution was evaporated to dryness under reduced pressure. The residue was taken up in water, neutralized, and adjusted to a convenient volume. These solutions were termed "stage 1 extracts" and were used for routine assays or as starting material for attempts at further isolation. When stage 1 extracts were adjusted to pH 2 to 3 with hydrochloric acid and extracted with diethyl ether all of the activity was found in the ether phase. The ether fraction was evaporated to dryness under reduced pressure and the residue taken up in water.

Further fractionation of the ether residue was accomplished on Dowex-1 (a weak anion exchange resin) chromatographic columns using formic acid as the eluent. Procedures for preparation of the columns and their gradient elution were adapted from those described by Hurlburt, et al (16). For bioassay, pooled fractions of the column effluent, representing a peak or group of peaks (peaks were identified by ultraviolet absorption measurements on each effluent fraction) were concentrated to a small volume under reduced pressure, and then taken to dryness in a vacuum desiccator. The residue was then taken up in water for assay.

Results

Isolation and Activity of Factor A: When the pooled fraction from the first satisfactory column experiment were assayed, it was found that the peak which inhibited Penicillium showed prominent absorption maxima at 227 and 286 mμ with a minimum at 255 mμ. The active peaks from several column runs (representing about 2.5 kgm fresh weight of plant material) were combined, reduced to dryness, taken up in water and extracted with ether. On concentrating the ether to near dryness and allowing it to stand overnight, a mass of needle-shaped crystals was obtained. It was found that these crystals were slowly soluble in hot water and on being recrystallized twice from water, 3.8 mg of colorless crystals were obtained. These crystals melted at 152 to 155° C and their ultraviolet absorption spectrum is shown in figure 1. The isolation was repeated on combined column effluents representing 3.5 kgm fresh weight of plant material. Fifty mg of crystals with similar properties (m.p. 152 to 153° C) were obtained in this attempt. An average value of 0.37 per mg per liter was taken for the specific extinction at 286 mμ. These crystals inhibited the growth of Penicillium by 0.11 % per mg per liter of medium and were also quite active in the corn borer assay. On the basis of these results, the substance was named "Resistance Factor A" (RFA).

The original 50 mg of RFA was sufficient for preliminary assays, elemental analysis, infrared absorption analysis and other tests, but for further characterization much larger quantities were required. By this time the bioassay procedures had been standardized to a higher degree and variations in the activity of stage 1 extracts were no longer ascribed only to variations in the assay procedures. As a result the charcoal adsorption and elution procedures were suspect and the decision was made to attempt to isolate the factor without utilizing charcoal.

The water extract of 25.3 kgm of whole corn plants (19 to 24 inches extended heights) was concentrated to 4.45 liters (A-50 concentrate; 5.52 gm/ml). A 500-ml portion of this concentrate was acidified to pH with HCl and subjected to continuous ether extraction in a Kutscher-Stendel apparatus. When the ether was evaporated to dryness, a dark-brown tarry residue containing needle-shaped crystals similar to those of RFA was obtained. No attempt was made to further purify this residue by ion-exchange chromatography, rather it was subjected directly to crystallization procedures. Crystals were obtained from an aqueous solution of the residue and,

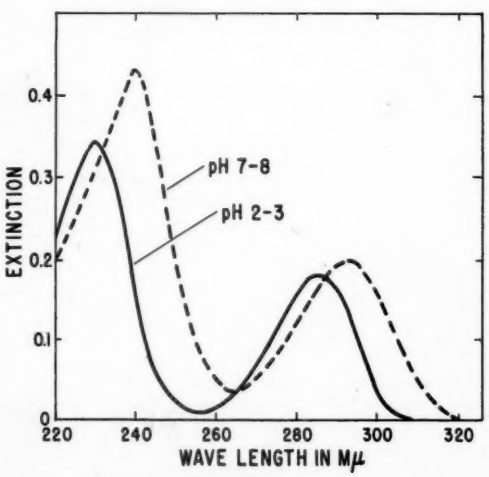

Fig. 1. Ultraviolet absorption spectrum of Resistance Factor A in aqueous solutions.

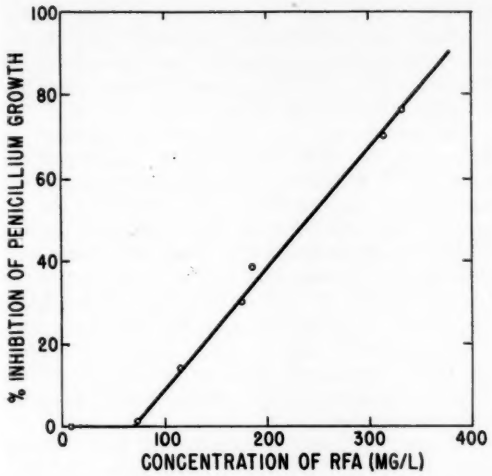

FIG. 2. Inhibition of Penicillium growth by Resistance Factor A in standard assays.

in addition, it was found that the residue was soluble in hot chloroform and that crystals could be obtained by diluting the chloroform with petroleum ether. By a combination of the two crystallization procedures, 180 mg of crude crystals with properties similar to those of RFA were obtained. The process was repeated with two other 500-ml portions of the A-50 concentrate with yields of 307 and 295 mg. The best yield (307 mg) represented a recovery of 0.11 mg per gm fresh weight of tissue.

When samples of the crystalline RFA obtained from the A-50 concentrate were assayed with both corn-borer and Penicillium good activity was obtained. The response curve for Penicillium is shown in figure 2 and is very similar to that obtained with corn borer larvae (11). It now became desirable to have an approximate value of the activity per mg of RFA in order to estimate the quantity of it in plant extracts. For this purpose, the average activity (0.18 % inhibition per mg per liter) obtained with concentrations of a Factor A greater than 100 mg per liter was used. By always assaying sufficient material to give more than 20 % inhibition in a standard assay the effect of the threshold level was reduced and the average activity value became a useful quantity.

CHEMICAL IDENTITY OF RESISTANCE FACTOR A: Preliminary steps towards the characterization of RFA were made and this work was completely by a group in the Department of Pharmaceutical Chemistry at the University of Wisconsin. The identity of RFA has been established as 6-methoxy-2(3)-benzoxazolinone ($C_8H_7O_3N$) (23) and methods of synthesis have been reported (24).

OTHER INHIBITORY SUBSTANCES IN AQUEOUS CORN EXTRACTS: When the aqueous phase from the ether extraction of the aqueous concentrates was assayed with Penicillium it was found to have high inhibitory activity. This observation coupled with the fact that all the activity of stage 1 extracts was readily soluble in ether indicated the occurrence of additional inhibitors which were lost or destroyed during the charcoal absorption and elution steps.

For example, in standard 50-ml Penicillium assays the A-50 concentrate averaged 6.7 % inhibition per ml. The best yield of RFA which had been obtained from the A-50 concentrate by ether extraction and recrystallization was 0.61 mg per ml which would account for 2.1 % inhibition per ml. In order to obtain another estimate of the RFA content of the A-50 concentrate, one ml of the concentrate was chromatographed using the Dowex-1 formic-acid system. Using the specific extinction value previously calculated, the peak due to RFA was estimated to represent approximately 1.0 mg of the crystalline material per ml of the concentrate. Even assuming this higher figure of 1.0 mg to be correct, RFA still only accounted for 3.5 % inhibition per ml or about one-half of the activity present in the A-50 concentrate. Small portions of the aqueous residue were chromatographed in the same manner and no traces of a peak similar to that ascribed to RFA were detected.

When a larger sample of the aqueous residue was chromatographed and the fractions pooled into three groups, the activity was found to be nearly equally divided between the first two groups (eluted by water and 1 *N* formic acid, respectively) and there was no activity in the third group (eluted by 1 to 4 *N* formic acid) where RFA should have appeared. Thus there were one or more substances other than Factor A in aqueous extracts of corn plants which inhibited Penicillium. This other substance or substances, since it also inhibited the corn-borer was termed Resistance Factor B (RFB). Table I summarizes the experience with the A-50 concentrate.

The charcoal adsorption procedure was reinvestigated to determine where Resistance Factor B was being lost. Alternative charcoal elution procedures were tried with no improvement over the one which had been in use. All the fractions which were discarded during the isolation were inactive; however, it

TABLE I

FRACTIONATION OF THE A-50 CONCENTRATED AQUEOUS EXTRACT OF CORN PLANTS. (INHIBITION OF PENICILLIUM IN STANDARD 50-ML ASSAYS)

FRACTION	% INHIBITION/EQUIV ML OF CONC	% INHIBITION/GM F.W. CORN TISSUE
Aqueous concentrate	6.7	1.20
RFA (assume 1.0 mg/ml aqueous conc)	3.5	0.66
RFB (aqueous residue after ether extraction of the conc)	3.6	0.64
Aqueous residue after fractionation on Dowex-1:		
Group 1	2.4	0.43
Group 2	2.1	0.37
Group 3	0.0	0.00

was not practical to assay the charcoal directly for RFB. In every case all the Penicillium inhibitory activity in stage 1 extracts was ether-soluble and it did not appear that RFB was eluted from the charcoal. The possibility exists that RFB was inactivated or altered to an ether-soluble form while adsorbed on the charcoal. While conclusive evidence that RFA was the only ether-soluble inhibitor in stage 1 extracts was not obtained, it was shown to account for the major portion of the activity of such extracts. An additional resistance factor (RFC) has subsequently been detected in portions of older corn plants and will be reported (7). RFC is ether-soluble but does not appear to have contributed to the activity of stage 1 extracts.

Distribution of Resistance Factor A in Developing Corn Plants: Considerable effort was expended to measure the RFA activity in corn plants of various stages of development. All of the attempts were based on Penicillium assays of stage 1 extracts (RFA) and the work was completed prior to the isolation of RFA and the detection of RFB. Results were variable and those reported here are considered to be of a preliminary nature.

The plants used in this phase of the study were field-grown plants of the inbred lines W 210 D and W 204. Samples were collected as the plants reached extended heights of 11, 16, 21, 26, 31, 40, 50, and 60 inches. Samples of plants under 40 inches were prepared and assayed for RFA activity on a whole-plant basis, while the larger plants were divided into different parts in order to determine the RFA distribution during growth stages normally attacked by the European corn borer. The two varieties showed no marked differences in either growth rate or fresh weight per plant.

Whole plant assays showed that the total activity per plant increased as the plants increased in size (table II) indicating a continued synthesis and accumulation of RFA in the growing plants. The RFA concentration (RFA activity/gm tissue) tended to decline as the plants grew taller.

From the standpoint of resistance to European corn borer establishment and survival, analyses of whole plants are of minimum significance. The borer does not feed on the plant indiscriminately, but typically attacks only certain tissues. Corn plants, even undersized inbreds, are not usually subjected to borer attack until the plants are well above 30 inches extended height, a fact which also minimizes the value of comparisons of small plants.

The results obtained with various portions of older plants are shown in table III. In general the highest levels of activity per gram fresh weight was found with leaves; sheaths, upper portion of the developing stem and the tassel were intermediate; and the lowest activity levels were found with lower stem portions prior to flowering and with all portions of the fully developed stalk at flowering. Those plant parts which are attacked by borer larvae are indicated in the table and the assay data for these tissues show varietal differences which may be of importance in respect to their comparative resistance to borer establishment.

The only attempts at assaying the roots of corn plants were carried out on seedling plants. Activity per gram fresh weight in the aerial portions ranged from four to six times that in the roots. Dark-grown seedlings, as did roots, had quite low levels of activity but the exposure of such seedlings to incandescent light during the last 12 hours of a seven-day germination period increased their activity four-fold.

Discussion

There is no deficiency of substances of plant origin which have insecticidal properties, but attempts to show a relationship between plant resistance in any particular case and a specific toxic substance within the plant are almost completely absent from the literature. Roach (22) obtained evidence that the resistance of apple trees to the wooly apple aphid, *Erisoma langerum* (Hausm.) was probably due in part to a chemical substance insoluble in alcohol and ether and only slightly soluble in boiling water. Some of the chemical factors contributing to the resistance of Solanum spp. to larvae of the Colorado potato beetle, *Leptinotarsa decemlineata* Say, have been isolated and characterized (18).

The evidence presented in this paper indicates that aqueous extracts of corn plants contain substances which inhibit the growth of Penicillium and the European corn borer when added to their synthetic diets.

Table II

Accumulation of RFA in Whole Corn Plants of Two Varieties 11 to 60 Inches Extended Height. (Activities Expressed as % Inhibition of Penicillium Growth in Standard 50-ml Assay)

Extended ht of plant, inches	W 210 D			W 204		
	Fresh wt, gm	RFA activity per plant	RFA activity per gm fresh wt	Fresh wt, gm	RFA activity per plant	RFA activity per gm fresh wt
11	11	5.9	0.54	11	11	1.10
16	14	8.8	0.63	12	8.8	0.73
21	30	13	0.43	26	20	0.76
26	54	22	0.41	49	24	0.49
31	96	36	0.37	82	30	0.37

TABLE III

RESISTANCE FACTOR A ACTIVITY OF STAGE 1 EXTRACTS OF DIFFERENT TISSUES OF TWO FIELD CORN INBREDS AT DIFFERENT STAGES OF GROWTH. (ACTIVITY EXPRESSED AS % INHIBITION OF PENICILLIUM GROWTH/GRAM TISSUE IN STANDARD 50-ML ASSAYS)

EXTENDED HT, INCHES	AV TASSEL RATIO	PLANT PORTION	RFA ACTIVITY			
			FRESH WT BASIS		DRY WT BASIS	
			W 210 D	W 204	W 210 D	W 204
40	40	Expanded leaves	0.71	1.04	4.4	5.9
		Expanded whorl leaves	0.20	1.10	1.2	5.8
		Rolled whorl leaves*	0.36	0.45	2.5	2.6
		Leaf sheaths	0.05	...	0.6	..
		Lower internodes	0.10	0.18	1.4	2.3
		Upper stem and tassel	0.61	0.18	8.0	1.9
50	59	Expanded leaves	0.33	0.30	1.9	1.5
		Expanded whorl leaves	0.51	0.75	2.5	3.6
		Rolled whorl leaves*	0.45	0.19	3.1	1.0
		Leaf sheaths	0.08	0.18	0.9	1.5
		Lower internodes	0.04	0.04	0.6	0.4
		Upper stem and ear shoots	0.26	0.18	3.4	2.4
		Tassel*	0.14	0.11	1.2	0.9
60	93	Upper leaves	0.87	1.10	3.3	3.9
		Middle leaves	0.48	1.03	2.3	4.0
		Lower leaves	0.52	0.90	2.8	4.0
		Leaf sheaths*	0.40	0.05	2.4	0.3
		Middle internodes*	0.36	0.01	2.9	0.2
		Upper internodes*	0.04	0.02	0.3	0.1
		Ear shoots*	0.24	0.17	2.2	1.4

* European corn borer feeding occurs on this tissue at this growth stage.

The effects of the inhibitors on the larvae are distinguishable from those of nutritional deficiencies (5). That the substances are actually toxic to the corn borer and do not act simply as feeding deterrents is shown by the fact that more borer larvae became established and fed on a diet containing an active corn leaf extract than did on the control diet lacking the extract (10). Neither the corn borer nor its host plant are stable biological entities while the borer is feeding on the plant. The orientation and feeding habits of the borers are influenced by negative phototaxis, positive thigmotaxis and saccharotrophism (4). In addition, its nutritional requirements vary from one stage of larvae growth to another (5). The response of the borers to these as well as other physical and chemical characteristics of the corn plant influences the tissue and portion of the plant on which the borer feeds, and hence, since the various plant portions have been shown to vary in their inhibitor content, will influence the degree to which the borers are exposed to the toxic substances. It was found for example that the inhibition of larval growth by RFA was inversely related to the sugar content of the medium on which the borers were reared. Thus sugars served to protect the borers from the action of the inhibitor and the saccarotrophic response of newly hatched borer larvae resulted in the larvae feeding on tisues with relatively high sugar content where they should be able to tolerate higher levels of RFA (6).

It is not yet possible to show that Resistance Factors A and B are toxic to corn borers feeding on the intact corn plant. While RFA appeared to be a rather stable compound, it may be only a fragment of a larger active or inactive substance which breaks down during extraction. One method of gathering evidence on the in vivo role of RFA and RFB is to determine the correlation between the mortality of larvae feeding on intact plants and the content of the inhibitory factors within the plants. The distribution studies discussed in this paper were an effort in that direction but they unfortunately dealt only with RFA. Since RFA and RFB appear to be equally involved and other inhibitory materials (e.g., RFC) are also involved, it is not anticipated that quantitative measurements of any one factor alone will be directly related to larval survival.

The resistance factors inhibit a wide range of microorganisms. Assay media containing sufficient RFA or RFB to completely inhibit the growth of *P. chrysogenum* remained free of bacterial and mold growth even after exposure to the air in the laboratory. The possibility that the resistance factors discussed here may also be involved in the resistance of corn to the attacks of other insects and fungi is of interest and worthy of further consideration. The resistance displayed by corn to the stalk-rot fungus, *Diplodia zeae* (Schw.) Lev., was reported by Johann and Dickson (17) to be related to an ether- and sap-soluble toxic substance present in the corn plant. It seemed likely that their extracts contained the inhibitors described above. Crystalline RFA and crude

preparations of RFB both inhibited the growth of *D. zeae* on synthetic agar medium.

Virtanen and Hietala (26) reported on the isolation of a substance from rye seedlings which inhibited *Fusarium nivale* in pure culture. This substance was characterized as 2(3)-benzoxazolinone, $C_7H_5O_2N$. After being informed of our results, Virtanen and coworkers (27) reported the isolation of a similar antifungal factor from wheat and maize. They speculated that this second factor was the 6-methoxy derivative of 2(3)-benzoxazolinone ($C_8H_7O_3N$). It has been shown by Smissman et al (23) to be this compound. Virtanen's two antifungal factors inhibit *Fusarium* sp., *Penicillium* sp. and a wide range of other micro-organisms in the same concentration range that RFA affects *P. chrysogenum* and the corn borer, i.e., complete inhibition at about 400 mg per liter.

Summary

The studies reported here are concerned with the isolation and distribution of corn-borer inhibitory substances present in corn plants. Several such substances were detected in aqueous extracts of corn plants and quantitative estimations of their activity were obtained by utilizing bioassays involving the fungus *Penicillium chrysogenum* and the European cornborer.

About one-half of the activity shown by aqueous extracts was due to an ether-soluble substance termed Resistance Factor A (RFA). This factor was isolated and found to be 6-methoxy-2(3)-benzoxazolinone.

RFA accumulated in the corn plant throughout its development. Leaves contained the highest levels of the factor and mature portions of the stem contained very low levels. The distribution of RFA showed a relationship to the patterns of high larval mortality which has been observed for borers feeding on the leaves of young corn plants. The concentration of RFA alone (ca 0.2 mg per gm FW) which was found in young corn plants was sufficient in synthetic diets to inhibit completely the growth of corn-borer larvae.

The ether-insoluble residues of aqueous concentrates contained an additional corn-borer inhibitor (or inhibitors) termed Resistance Factor B (RFB) which accounted for the other half of the activity shown by such concentrates.

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PROPERTIES OF A VOLATILE FRACTION FROM UREDOSPORES OF PUCCINIA GRAMINIS VAR. TRITICI AFFECTING THEIR GERMINATION AND DEVELOPMENT. I. BIOLOGICAL ACTIVITY[1,2]

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Previous studies on stem rust uredospores (1) showed that germination was prevented by increasing the number of spores floated in a confined space, and that the inhibition of germination was caused by substances emanating from the spores and accumulating in the liquid on which they floated. There were indications that the active material might be volatile. The present experiments emerged from attempts to obtain a germination inhibitor in purer form from the crude aqueous solutions. Unexpectedly, these attempts led at once to the preparation of a volatile fraction which caused an increase in rate of germination rather than an inhibition. Further studies of this fraction showed that it was also capable of inducing on aqueous solutions the formation of structures closely resembling the infection structures formed during the early stages of development on a host plant. In the following report are presented some of the important biological properties of this volatile fraction.

MATERIALS AND METHODS

Uredospores of Race 56 of stem rust of wheat, *Puccinia graminis* var. *tritici*, were used for all assays and for all preparations of active materials. Spores were collected and stored as reported previously (1).

Germination and development of the germ-tubes was studied with spores floated on aqueous solutions. Except as shown otherwise, the solutions were buffered with 0.01 or 0.005 M potassium phosphate buffers. The test spores were floated on water and a loopful transferred at once to each test solution. The test vessel was covered, sealed with vaseline, and incubated in darkness at 18 to 20° C.

[1] Received March 1, 1957.
[2] This work was supported in part by a contract with the Chemical Corps, Fort Detrick, Frederick, Maryland. Concurrent but independently conducted studies by French, Massey, and Weintraub on the same problem are reported as Part II.

The vessels used for the most critical observations were Pyrex micro-Conway diffusion dishes (3) having a total volume when sealed of 5 ml and with a center well with 0.8-ml capacity. Test solutions were placed in both the center well and the outer ring, or in the outer ring alone when diffusion through air was required for the test substances to reach the spores. Spores were floated on 0.5 ml of liquid in the center well, and the vessels were covered with a polyethylene cover. Rectangular vessels made of plexiglas and covered with a coverslip were also used extensively. Pyrex and plexiglas vessels have been found to release lesser amounts of stimulatory materials than soft-glass vessels or Van Tieghem vessels cemented with sealing wax. For short-term tests interference by these contaminants is small, but for longer-term incubations it is sometimes sufficient, with the Van Tieghem vessels, to mask completely the true effects of a test solution.

Records of germination and germ-tube growth were usually made at 2 1/4 hours after the spores were floated. Germination was estimated as reported previously (1). Germ-tube growth was estimated in terms of spore length, which is quite uniform (35 μ), and was then converted to absolute units. The differentiation of germ-tubes was not definitive in less than 12 hours, and was therefore recorded only after incubation for at least 15 hours.

Crude inhibitor solutions were prepared in pyrex vessels by floating uredospores for 5 to 7 hours on double-distilled water in diffuse light or in darkness, using approximately 3 gm of air-dry spores per liter. The temperature during incubation was not controlled, and varied from 20 to 29° C. Aeration was accomplished by occasional shaking of the closed vessel, or by continuous agitation with a magnetic stirrer and a gentle stream of air. In the latter type of preparation, the stream of air then passed through a series of cold traps maintained at temperatures from + 1° C down to − 60° C. At the end of an incubation the spores were allowed to settle and the solution was

withdrawn by gentle suction, passing through a small wad of non-absorbent cotton to remove any spores still in suspension. A clear, light-yellow filtrate was obtained having a pH of about 6.0.

Results

Effects on Germination of Fractions Derived from Solutions of Crude Inhibitor: The crude inhibitor solution was distilled at 100° C and atmospheric pressure and a series of fractions of the condensate collected in an ice-bath. None of the distillate fractions inhibited, but instead some of them stimulated the germination of uredospores. In small samples of spores (about 1,000 spores/ml), where self-inhibition is slight, the stimulation was not always striking, but with larger populations the distillate induced rapid and often complete germination under conditions which permitted no germination at all in the controls. The distillates therefore overcame the natural self-inhibition (1). Most of the activity was found in the first 10 to 20 % distilling over.

To determine whether a volatile stimulant was simply being removed and concentrated from a mixture of stimulant and inhibitor, distillate and residue were recombined, thus diluting both components back to the original volume of crude inhibitor. The combination no longer possessed the inhibitory action of the original crude inhibitor (table I, left hand column). This loss could not be ascribed to the altered pH, since a second series of tests with the same preparation buffered at pH 7.0 (table I, right hand column) likewise showed a loss of inhibitor activity in the recombined residue and distillate. Since neither the residue nor the combination of residue and distillate showed evidence of inhibition in the buffer series, the process of distillation apparently resulted not only in the separation or formation of a volatile stimulant, but also in a loss of the original inhibitory activity. The inhibition shown by the unbuffered residue was different from that of the crude inhibitor, since unlike the latter it was not evident in phosphate buffer. Furthermore the inhibition by the residue was abolished by low concentrations of distillate which were insufficient to overcome the action of the crude inhibitor. The activity of the stimulant could be demonstrated at higher concentrations by its counteraction of the self-inhibitor and, as indicated in table I, was readily detected by its stimulation of germination on potassium phosphate at neutrality.

Table I

Germination of Fresh Uredospores at 70 Minutes on Crude Inhibitor and on Fractions Obtained from It by Distillation

Preparation tested	Distilled water		Potassium phosphate, 0.01 M, pH 7.0 †
	pH	% Germination	% Germination
Control	6.0	90	10
Crude inhibitor *	6.0	2	0
Residue **	7.4	2	40
Distillate **	6.0	95	40
Residue + distillate **	7.4	80	50

* A 2-fold dilution of a preparation obtained in distilled water.

** Diluted to give concentration equivalent to that of the crude inhibitor.

† The pH of the test solutions was maintained constant by this buffer.

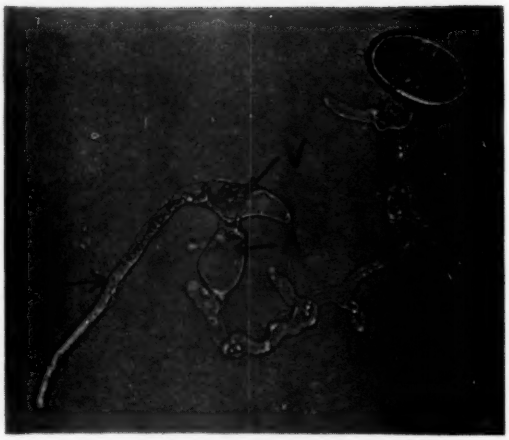

Fig. 1. Infection structures developed from a uredospore. Typical individual from a sample floated on 0.005 M potassium phosphate, pH 7.0, for 2 days. A, appressorium; V, substomatal vesicle; I.H., infection hypha. The bulk of the protoplasm was earlier concentrated in the appressorium, has now migrated into the vesicle and infection hypha.

Using the increase of germination in neutral potassium phosphate as an index of activity, it was found that some stimulant was recovered in the cold traps through which air was circulated during the preparation of crude spore eluates, but most of the activity was recovered only after distillation. Comparable yields were obtained by distillation at 100° C, or at 40 to 50° C with reduced pressure. The stimulant was obtained by distillation from water and from solutions buffered at pH 5.5 or at 7.5.

Effects of Distillate on Growth and Differentiation of Germlings: Stimulation of germination by distillates was accompanied at the higher concentrations by other effects upon development. Growth in length of the germ-tubes was retarded and their usual polar growth gave place to the formation of apical swellings. These swellings first appeared several hours after exposure to the distillate. Into them the orange-yellow contents of the spore and hypha emptied as the swellings enlarged. Each swelling later gave rise to a secondary protuberance into which the protoplasm of the first vesicle migrated. These secondary swellings then elongated, sometimes perpendicular to, sometimes in the direction of the hyphal axis. From this second swelling a filament grew out

to a length usually not exceeding 200 μ. The sequence of formation, the physical relationships and the form of these three structures (fig. 1) resembled the appressoria, sug-stomatal vesicles, and infection hyphae which form during infection of the wheat plant (5). They were very similar in appearance to structures formed on non-living substrates and identified as appressoria, vesicles, and infection hyphae of *P. graminis* var. *tritici* by Hurd-Karrer and Rodenhiser (9). These authors reported similar structures formed on agar by several other cereal rusts. Sharp and Smith (10) also reported such structures forming in large numbers from uredospores of *P. coronata*, and Dickinson (4) has observed them on *P. triticina* hyphae formed from spores germinated on artificial membranes. The property of inducing formation of these structures is the most distinctive activity so far observed of the distillates from uredospore extracts. In the most favorable circumstances, up to 80 % of all spores in a preparation containing the distillate formed vesicles and infection hyphae; without distillate, a typical vesicle and infection hypha were rarely formed.

The activity of the distillate was obtained in Conway dishes either by direct contact of the solution with the spores or by diffusion. Applied in either way, equal amounts of distillate produced similar development of infection structures, but equal stimulation of germination, if determined over short intervals after floating the spores, required slightly more distillate when the only access to the spores was through the gas phase.

Effect of Distillate Concentration: The effect of distillate concentration is shown in table II for media with and without potassium phosphate buffer. All data in this table were obtained with a batch of distillate containing the volatile materials from 2 gm of spores in 15 ml of condensate, and the experiments were done with the same batch of test spores over a period of 7 days. The test spores had been in storage for about 2 months. On each medium, an increase in concentration of distillate increased the rate of germination. On phosphate at pH 7.0, but not at pH 6.0, the stimulation of germination declined again at higher concentrations of distillate.

On distilled water and on potassium phosphate buffers, the growth of germ-tubes was inhibited by the highest concentrations of distillate (table II). Since potassium phosphate also strongly inhibited the growth of germ-tubes, the reduction of germ-tube growth by the distillate was generally less striking on these buffers. Inhibition by the buffer was found with similar concentrations of potassium acetate, and was completely prevented by the addition of $CaCl_2$ or Ca acetate to give a final concentration of 10^{-4} M Ca^{++} (with 10^{-2} M potassium salt). In the presence of both Ca^{++} and K^{+}, inhibition by the distillate occurred only at high concentrations, resembling its action when added to distilled water.

On the higher concentrations of distillate, terminal swellings formed soon after germination, before the hyphae elongated very much. At the highest concentration, tested only at pH 7.0, all development ceased without differentiation of additional structures from these swellings.

At or near the lowest concentration which inhibited growth of the germ-tubes, the maximum differentiation of infection structures occurred (table II). Swellings which developed into characteristic appressoria, vesicles, and infection hyphae were elicited both with and without added potassium phosphate. Variability in the frequency of vesicle formation has not yet been completely eliminated, and there is evidence that some other factor is required for the optimal effects of the distillate. At pH 7.0 better development of infection structures was supported by 0.005 M potassium phosphate than by 0.01 M. On a medium containing Na, Cl, and Si plus the elements known to be essential for fungus growth, all in low concentration, still better and more uniform production of vesicles has been observed, but it has not been possible to demonstrate a clear effect of any component of this medium. Neither Zn nor Ca had any clear effects on vesicle formation, but the long hyphae formed in the presence of Ca made it more difficult to detect vesicles except with the highest concentrations of distillate.

Up to an optimum, increasing concentrations of distillate increased the frequency of terminal swellings

Table II

Effect of Distillate Concentration on Germination, Germ-tube Growth, and Development of Infection Structures

Concentration of distillate *	Germination in 2½ hrs, %	Germ-tube length in 2½ hrs, μ	Infection structures 18 to 19 hrs **
	Water, pH ca 6.0		
0	25	200	–
0.04	50	200	±
0.10	*80*	200	+++
0.20	*80*	200	(+++)
0.50	60	80	(++++)
	Potassium phosphate, pH 6.0		
0	15	80	–
0.04	40	150	±
0.10	50	140	+++
0.20	70	80	(++++)
0.50	*90*	40	(++++)
	Potassium phosphate, pH 7.0		
0	10	40	–
0.04	60	25	–
0.10	*75*	40	(+)
0.20	30	20	(+++)
0.50	10	10	(+++)
0.80	5	8	(+)

* As fractions of the original concentration of this preparation.

** Symbols indicate approximate % of spores with swellings which later developed into characteristic infection structure. None, –; less than 1 %, ±; a few %, (+); about 10 %, (+++); more than 50 %, (++++).

TABLE III

EFFECT OF DISTILLATE ON DIFFERENT SPECIES OF RUSTS

SPECIES	SPORE TYPE	RESPONSE *		
		GERMINATION	GERM-TUBE GROWTH	INFECTION STRUCTURES
Puccinia graminis var. *tritici*	Uredospores	+	–	++
P. coronata	Aeciospores	0	–	0
P. caricina	Aeciospores	+?	–	+
Uromyces phaseoli	Uredospores	+	–	+
Gymnoconia insterstitialis	Aeciospores	–	–	0

* All tests on 0.005 M potassium phosphate, pH 6.0; stimulation, +; inhibition, –; no definite effect, 0.

and of infection structures. Above this concentration, there were further increases in the percentage of germ-tubes forming swellings, but there was also a gradual retardation of the further development of these swellings. High concentrations of distillate inhibited particularly the elongation of infection hyphae.

In other series, using different preparations of distillate, similar results were obtained. Comparison between different preparations was made on the assumption that a certain level of germination was achieved at similar concentrations of stimulant. Calculating the relative concentrations of two samples from one level of germination and adjusting the germination-concentration curves on that basis gave remarkably good agreement in the effects of all other concentrations. Furthermore, the concentrations of stimulant then found to elicit differentiation of infection structures were quite similar for different preparations of distillate. These comparisons have not been carried far enough, however, to afford clear evidence that the different activities reside in a single substance.

EFFECT OF DISTILLATES ON OTHER RUST FUNGI: A few exploratory tests were made to find whether the volatile fraction had any effect on the development of other rust spores. With all of the spores tested, there were clear effects of the distillate, although the nature of the effects differed, ranging from inhibition of germination to stimulation of vesicle formation (table III). These differences may very well represent differences in sensitivity, since only concentrations known to effect vesicle formation in *P. graminis* var. *tritici* were used in these tests.

DISCUSSION

The substances released from the cells of an obligate parasite and affecting its development are likely to be of importance to the problem of artificial cultivation of these fungi. Such substances are also of potential importance to an understanding of the interaction between host and parasite. The volatile fraction discussed in this paper seems of particular interest in the latter connection, because it simulates the action of the host plant in causing the differentiation of infection structures. Thus a solution containing relatively few substances (possibly only one crucial substance) is able to elicit development of a series of structures whose formation on the host plant is strictly correlated with the position of guard cells, stomatal openings and sub-stomatal chambers. The formation of structures characteristic of the growth of the parasite on the host and needed for successful infection does not, therefore, require any special physical stimuli, but can be brought about in spores floating on water in the presence of specific chemical substances. That an occasional spore develops infection structures in the absence of added stimulant (9) suggests that the supply of this substance is occasionally high enough in a spore to permit the differentiation of germ-tubes.

There is no good evidence from these experiments indicating whether the distillate contains one or more substances influencing spore development, but in view of the method of preparation, it seems unlikely that it contains more than a few substances at most. The observed differences in activity at different pH's and with and without potassium phosphate do not exclude the possibility of a single active substance.

Although some of the volatile stimulant appears to be released by metabolizing spores, the bulk of the activity obtained in these experiments was derived from substances first released by the spores into the aqueous phase and then converted in the absence of spores into a volatile stimulant. The conversion takes place rapidly during distillation at 40° C or above, and probably occurs slowly on standing at or even below room temperature. The stimulant must be different from the germination inhibitor since its addition to a crude inhibitor solution never enhances but may abolish inhibition, and since it counteracts the self-inhibition in large populations. It is probably different also from the stimulant of germ-tube growth suggested by Ezekiel's (6) and Yarwood's (11) experiments, because its primary action on germ-tubes is to inhibit their growth. Only in exceptional circumstances and in the absence of Ca does it increase germ-tube growth. There is no resemblance in odor between our preparations and trimethylethylene, which Forsyth has claimed to be a natural inhibitor from uredospores of *P. graminis* var. *tritici* (7), nor does trimethylethylene stimulate germination under the test conditions employed in this investigation. On the other hand, the active preparations described in this paper have points of similarity with those described by French, Massey, and Weintraub (8), notably in their common property of stimulating germination and of eliciting the development of infection structures.

Many investigators have noted a distinctive odor associated with one or more stages in the development of rusts. Although it is not possible to identify odors by description, a fragrance analogous to that described

by several observers for other rusts (2) has often been detected in the distillates derived from stem rust uredospores. The fishy odor of fresh distillates frequently gives place upon dilution or storage to an odor more like the fragrance of flowers. If the odors and the biological activities of the preparations discussed in this paper are properties of the same compound(s), then the widespread production of odors by rusts may have some significance in the ontogeny of parasites of this group.

Summary

A fraction stimulating uredospore germination was obtained by distillation from the substances released into water by uredospores. The original water solutions which inhibited germination (crude inhibitor solutions) gave rise to the stimulant during distillation and at the same time lost their inhibitory activity. Some stimulant was apparently present in the original solution.

The activity of distillates in stimulating germination was demonstrable under a variety of conditions. It was most readily detected when an inhibition by some other agent was counteracted. Distillate overcame the self-inhibition of germination occurring in large populations of spores, and counteracted the inhibitions imposed by neutral potassium phosphate and by residues left after distillation of inhibitor solutions.

The same distillates affected the rate and extent of germ-tube growth and differentiation. Germ-tube growth was retarded under a variety of conditions, and at high concentrations was almost completely prevented. At concentrations similar to those retarding elongation, the germ-tubes developed a series of structures which have been identified as appressoria, substomatal vesicles, and infection hyphae. These structures formed in high percentage (up to 80 %) from spores floating on an aqueous solution.

Stimulation of germination, inhibition of germ-tube growth, and differentiation of infection structures were elicited at 18° C either by direct contact of the solution of distillate with the spores, or by diffusion from the solution across the air gap.

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PROPERTIES OF A VOLATILE FRACTION FROM UREDOSPORES OF PUCCINIA GRAMINIS VAR. TRITICI AFFECTING THEIR GERMINATON AND DEVELOPMENT. II. SOME PHYSICAL AND CHEMICAL PROPERTIES[1]

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Allen (1) demonstrated that the germinability of uredospores of *Puccinia graminis* var. *tritici* is influenced by an endogenous inhibitor from which they can be freed to large extent by floating on water. Spores treated in this way are referred to herein as "inhibitor-depleted" while the unwashed spores are designated "self-inhibited." The wash water, after removal of the spores, was shown to contain material which inhibited germination of inhibitor-depleted spores.

In the course of an investigation of the endogenous inhibitor, we have obtained evidence of the occurrence of a germination stimulator in the emanations from moist spores. This paper describes some of the physical, chemical, and biological properties of the

[1] Received February 12, 1957.

stimulator. A parallel, but independent, investigation by Allen is reported as Part I (2).

Methods and Results

The standard method for extraction of stimulator consisted of suspending spores in five times their weight of distilled water by brief agitation in a Waring Blendor, allowing the mixture to stand overnight at 4° C, and distilling it at atmospheric pressure with the receiver in an ice bath.

Among other procedures which gave germination-stimulating preparations were (a) suspension of spores in water followed immediately by distillation, (b) suspension and extraction as in the standard method but with removal of the spores prior to distillation of the extract, (c) vacuum distillation of dry or wet spores at room temperature with condensing traps at – 80° C, and (d) passage of moist air through a mass of spores and collection in traps at – 80° C.

Spores of race 56, collected by means of a cyclone device (3) from field-grown Baart wheat, were used for the most part; a number of other races also have been shown to be satisfactory sources of the stimulator.

Bioassay of Volatile Stimulator: Assays were carried out in Pyrex glass or porcelain Conway diffusion cells of approximately 30 ml volume. Four ml of the test solution were placed in the annulus and a relatively small number (of the order of 1000) of self-inhibited uredospores were floated on 2.5 ml of Sorenson's M/90 pH 6.0 phosphate buffer in the inner

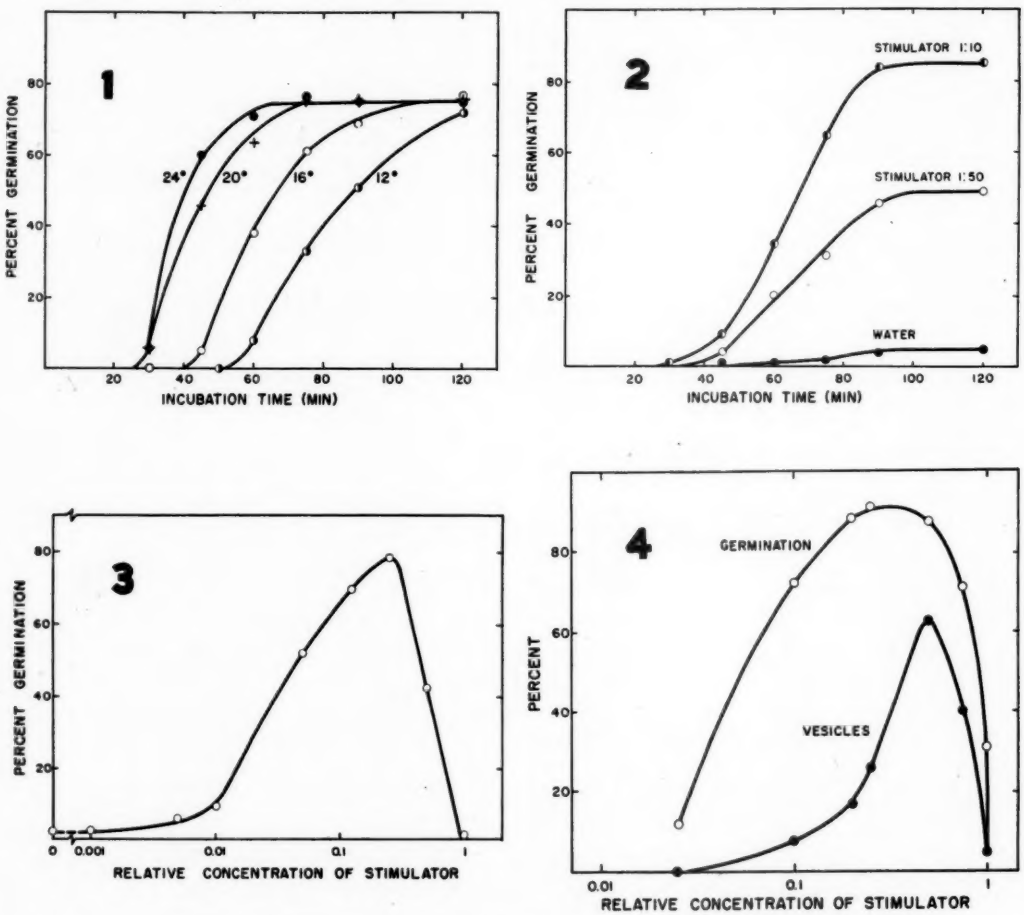

Fig. 1. Time-course of germination at optimal stimulator concentration as a function of temperature.

Fig. 2. Time-course of germination at two concentrations of stimulator. Temperature = 16° C.

Fig. 3. Stimulation of germination as a function of stimulator concentration. The highest concentration shown is that of the undiluted distillate. Temperature = 16° C. Incubation period 2 hrs.

Fig. 4. Concentration-response curves for germination and for vesicle formation induced by a single preparation. Temperature = 27.5° C. Incubation time 18 hrs.

compartment. The cell was closed with a ground glass cover and incubated in darkness at 16° C for 90 to 120 minutes after which germination was stopped by addition of formalin and one loopful of spores was removed for subsequent microscopic examination. Three groups of 100 spores, or a total of 300, were counted from each cell. A spore was considered as germinated if it had produced a germ tube of length greater than the germ tube diameter.

Spores of race 56 produced on Baart wheat in the greenhouse proved satisfactory as indicator spores and were used in the majority of tests. Spores of races 17 and 38 also were suitable. Old, as well as freshly harvested, spores were usable provided the viability remained high.

Because germination of Puccinia uredospores has been reported as sensitive to various environmental factors, several variants of the standard assay method were compared. Essentially identical results were obtained in Pyrex and in porcelain vessels. Whether the test solution or the test spores were introduced into the Conway cell first was immaterial. Germination under the assay conditions was not affected by diffuse daylight or fluorescent light of moderate intensity such as normally encountered in the laboratory. The number of spores used in the cell could be increased to at least three times the standard amount without significantly influencing the assay. Whether the test spores were floated on M/90 pH 6.0 phosphate buffer or on distilled water made no difference. Variations of the buffer pH within a few tenths of a unit were inconsequential.

The influence of incubation temperature on the time course of germination is shown in figure 1. Temperatures between 16° and 24° C were found suitable. Because of the large temperature coefficient of the germination process, care should be taken that all test solutions be brought to the incubation temperature prior to starting the assay.

Figure 2 shows that the general shape of the progress curve of germination is unaltered over a wide range of stimulator concentrations. In this experiment, germination had virtually attained its maximal value by 90 minutes of incubation.

Figure 3 illustrates the relation between concentration of the crude distillate and the germination response. The curve exhibits a maximum, there being an optimal concentration above which smaller stimulations and even inhibition may result.

Physical and Chemical Properties: The active material could be extracted from aqueous solution by a number of organic solvents including amyl acetate, benzene, carbon tetrachloride, chloroform, petroleum ether, and xylene. The stimulator could be removed completely from aqueous solution by addition of activated carbon.

It is apparent from the mode of preparation and bioassay of the stimulator that it is a substance of appreciable volatility. However, even prolonged boiling of aqueous solutions at atmospheric pressure did not remove all activity.

The volatile character of the stimulator permitted the utilization of a convenient and simplified technique for testing its chemical reactivity prior to isolation. Tests were carried out by treating stimulator solutions wth large excesses of selected reagents in stoppered Pyrex test tubes or sealed ampules and determining the residual activity of the reaction mixtures by bioassay in the Conway cells. Volatile acids or alkalies were immobilized by appropriate adjustment of pH prior to bioassay. Creeping of alkaline reaction mixtures was prevented by coating the rim of the inner compartment very lightly with lanolin. Reagent controls were included in all cases.

The stimulatory activity of extracts was not diminished by treatment with sulfuric acid at concentrations at least as great as $2\,N$ or with potassium hydroxide up to $3.5\,N$, providing evidence that the stimulator is not of acidic or basic character. The active compound is highly thermostable, being unaltered after heating for 5.5 hours at 120° C in a sealed ampule in an autoclave. The stimulator was resistant also to hot acid and alkali, no change in activity being caused by prolonged heating in ampules at 120° C of solutions $2\,N$ in sulfuric acid or $3.5\,N$ in potassium hydroxide.

Results of tests with a variety of reagents used for characterizing organic functional groups are summarized in table I. The results are expressed as complete, or virtually complete, inactivation (loss greater than 95 %), partial inactivation (50 to 95 % loss), or no significant inactivation (less than 20 % loss). It should be borne in mind that the negative results are somewhat less informative than the positive effects inasmuch as the volatile nature of the stimulator precluded some of the reactions being carried out under optimal conditions of heating or refluxing.

None of several reducing agents had any effect on potency of the stimulator. Activity was destroyed completely by such oxidizing reagents as neutral permanganate, acidic dichromate, neutral or alkaline hydrogen peroxide, peracetic acid, bromine, fuming sulfuric acid, and Fenton's reagent. Potassium periodate did not diminish the activity, pointing to the absence of a glycol structure. No inactivation was caused by treatment with a number of sulfhydryl reagents (sodium nitroprusside, mercuric chloride, neutral silver nitrate) nor by picric acid which might be expected to react with an amino function.

Of the reagents that might be expected to react with an olefinic bond, iodine was ineffective whereas mercuric perchlorate (4) completely removed the activity. The activity could be regenerated by addition of lithium chloride to an aqueous solution of the inactive mercury complex (table II). Mercuric ion can react with a considerable variety of functional groups so that its inactivation of the stimulator is not to be taken as definitive evidence of a double bond. Similarly, the effect of reagents such as bromine and fuming sulfuric acid could be due to addition to an olefinic bond as well as to oxidation of other groups.

TABLE I

INACTIVATION OF STIMULATOR BY TREATMENT WITH CHEMICAL REAGENTS

REAGENT	INACTIVATION *
Oxidizing reagents	
$KMnO_4$ (neutral)	+
$K_2Cr_2O_7$ (acidic)	+
KIO_4 (acidic)	−
H_2O_2 (neutral)	+
H_2O_2 (alkaline)	+
Peracetic acid	+
Br_2	+
H_2SO_4 (fuming)	+
Fenton's reagent [H_2O_2, $Fe(NH_4)_2(SO_4)_2$, H_2SO_4]	+
Reducing reagents	
H_2	−
$SnCl_2$ (acidic)	−
$FeSO_4$	−
$Na_2S_2O_4$	−
$Na_2S_2O_3$	−
Na_2SO_3	−
Carbonyl reagents	
Hydroxylamine	±
Semicarbazide	±
Thiosemicarbazide	±
Dimedon	±
4-Nitrophenylhydrazine	±
as-Diphenylhydrazine	±
α-Naphthylhydrazine	±
2,4-Dinitrophenylhydrazine	±
α-Benzyl-α-phenylhydrazine	±
2,5-Dichlorophenylhydrazine	±
Tollen's reagent (ammoniacal $AgNO_3$)	±
Benedict's reagent (alkaline $CuSO_4$)	−
Hydroxyl reagents	
3,5-Dinitrobenzoyl chloride	±
α-Naphthylisocyanate	±
$FeCl_3$	−
Amine reagent	
Picric acid	−
Sulfhydryl reagents	
$Na_2Fe(CN)_5NO$	−
$HgCl_2$	−
$AgNO_3$ (neutral)	−
Olefin reagents	
I_2	−
$Hg(ClO_4)_2$	+

* Loss of 95 % or more of activity, +; loss of 50 to 95 % of activity, ±; loss of less than 20 % activity, −.

Partial loss of activity resulted from treatment with a considerable number of carbonyl reagents including hydroxylamine, semicarbazide, thiosemicarbazide, dimedon, Tollen's ammoniacal silver nitrate reagent, and several arylhydrazines. Benedict's reagent was without effect.

Partial inactivation was caused also by hydroxyl reagents such as 3,5-dinitrobenzoyl chloride, and α-naphthylisocyanate. Ferric chloride, a phenol reagent, was without effect.

TABLE II

INACTIVATION OF STIMULATOR BY TREATMENT WITH MERCURIC PERCHLORATE AND REGENERATION WITH LITHIUM CHLORIDE

FRACTION	% GERMINATION
Untreated stimulator	61
Mercuric perchlorate	2
Stimulator + $Hg(ClO_4)_2$	7
Stimulator + $Hg(ClO_4)_2$ + LiCl	41

Partial inactivation might result from either (a) an incomplete reaction between the reagent and stimulator or (b) the presence of two or more active substances differing in structure and reactivity. Although the available data do not distinguish unambiguously between these two possibilities, the rather consistent behavior of a large number of diverse reagents, such as the carbonyl reagents used, would seem to favor the latter alternative.

On the basis of the foregoing it may be suggested provisionally that the extract contains at least two germination stimulators, one possessing a carbonyl function, the other a hydroxyl function.

A derivative of the carbonyl-bearing stimulator was isolated in the following manner. Dishes of crude stimulator solution and of an aqueous solution containing semicarbazide and sodium acetate were placed in a closed desiccator. The volatile stimulator diffused into the reagent solution and was converted to a crystalline semicarbazone which accumulated on the surface.

The biologically active stimulator could be regenerated by acid hydrolysis of the semicarbazone. A small amount of the semicarbazone was mixed with 9 % phosphoric acid and distilled. As shown in table III, the distillate was highly active. Regeneration of the stimulator could be demonstrated also by placing semicarbazone and dilute phosphoric acid in the annulus of the Conway cell. As little as 1.5 μgm of the crude semicarbazone per vessel produced a significant stimulation when assayed in this manner.

STIMULATION BY KNOWN COMPOUNDS AND OTHER DEVELOPMENTAL EFFECTS: Germination of self-inhibited uredospores could be stimulated to some extent by a variety of substances including ethylene, furfuryl alcohol, furfuryl acetate, 2,4-dinitrophenol, and levulinic acid. However, none of these compounds,

TABLE III

FORMATION OF DERIVATIVE AND REGENERATION OF CARBONYL STIMULATOR

TEST SOLUTION	% GERMINATION
Water	6
Distillate from acidified semicarbazide reagent	6
Distillate from acidified semicarbazone derivative	91

TABLE IV

ANTAGONISM OF ENDOGENOUS INHIBITOR BY STIMULATOR (TEST SPORES WERE DEPLETED OF INHIBITOR BY FLOATING ON WATER PRIOR TO USE)

TEST SOLUTION	% GERMINATION
Water	80
Water + stimulator	97
Inhibitor *	10
Inhibitor + stimulator	97

* Inhibitor solution prepared by floating 200 mg spores on 20 ml distilled water at 16° C overnight.

even at its optimal concentration, was nearly as effective as the endogenous stimulator.

Prolonged exposure of spores to stimulator solutions at appropriate temperatures, induced, in addition to increased germination, the formation of structures resembling appressoria, infection hyphae, infection pegs, and substomatal vesicles. Definitive evidence that these responses were brought about by the same substance that stimulates germination is not yet available. Dilution curves for germination and for vesicle formation are shown in figure 4. The curves exhibit a measure of similarity but also appear to differ appreciably.

MECHANISM OF STIMULATION: Although the detailed mechanism is unknown, it is evident that the stimulator operates by overcoming the influence of an endogenous inhibitor. Thus, the self-inhibition exhibited by concentrated masses of spores on water or agar was abolished by addition of stimulator extract. Furthermore, the stimulator was shown to overcome the influence of endogenous inhibitor solution on inhibitor-depleted spores (table IV).

SUMMARY

Extracts which accelerate markedly the germination of rust uredospores were prepared from resting spores. The active material is relatively volatile and is soluble in water and various organic solvents. Although it has not yet been isolated or identified, evidence from inactivation tests with various chemical reagents suggests that there may be two active substances, one possessing a carbonyl group and the other a hydroxyl group.

The stimulator promotes germination by overcoming the self-inhibition due to an endogenous germination inhibitor. Under suitable environmental conditions crude preparations of the germination stimulator also induce differentiation of the germ tubes into structures resembling appressoria, infection hyphae, infection pegs, and substomatal vesicles.

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PHOTOCONTROL OF ANTHOCYANIN FORMATION IN TURNIP AND RED CABBAGE SEEDLINGS[1]

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Syntheses of the prominent red and blue anthocyanin pigments of many plant tissues either require radiation or are enhanced by it. The older work was discussed by Arthur (1) and Blank (3). Arthur (2) suggested that red color formation in apple skin after irradiation was greatest in the regions of 2900 to 3100 Å and 3600 to 4500 Å. More recently, Eddy and Mapson (8), Slabecka-Szweykowska (19, 20), Eberhardt (7), and Withrow, Klein, Price, and Elstad (21) studied anthocyanin synthesis in several plant tissues as influenced by radiation. Both Slabecka-Szweykowska and Withrow and his associates noted synthesis at wavelengths longer than 6000 Å.

The effectiveness of radiation for inducing anthocyanin formation in red cabbage seedlings, *Brassica oleracea* var. Red Acre, and turnip seedlings, *Brassica rapa* var. Purple Top White Globe, is reported here.

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MATERIALS AND METHODS

Seeds of red cabbage and turnips were germinated in darkness on three sheets of Whatman No. 2 filter paper moistened with water at 25° C in Petri dishes. The moistening solution for the turnip seed was an aqueous solution containing 32 ppm of chloramphenicol, which was observed to suppress chlorophyll formation and increase anthocyanin production in a manner similar to that reported for terramycin (14).

Seedlings were irradiated with a spectrograph source or with a fluorescent or incandescent source of radiation after 3 days of germination time, when the cotyledons had spread out after shedding the seed coats. They were then returned to darkness for 16

to 24 hours. This period was found to be adequate for anthocyanin synthesis induced by the radiation. Four or more replicates were used in the general irradiation experiments and duplicate lots for the spectrographic ones. Each type of experiment was repeated many times.

Analyses of the anthocyanin content of the seedlings were made at the conclusions of the dark periods. Counted numbers of seedlings were extracted in 0.01 molal HCl in aqueous 25 % 1-propanol. The seedlings were extracted overnight at 5° C and finally in a boiling water bath for 1 minute. Analyses were based on the differences in absorptions at 6200 Å before and after addition of Na_2CO_3 to make the solutions alkaline. This procedure permitted correction for scattering of light due to a small amount of suspended material. Adjustments were made in the volume of the extracting solution and in the number of seedlings used to give an optical density of 0.1 to 1.0 where possible. The optical density values were transformed to concentrations by using the molecular extinction coefficient of 2.45×10^4 at 5350 Å, for cyanidin (18). A factor was used to convert the optical density values determined at 6200 Å in the presence of Na_2CO_3 to those at 5350 Å for cyanidin in acid solution. This procedure does not distort the relative scale and gives an approximate estimate of the absolute values, which depend on the conditions chosen for growing the seedlings.

Experimental lots of seedlings were irradiated with a bank of white fluorescent lamps giving a maximum illumination of 1,000 ft-c and an irradiance for anthocyanin synthesis at 7000 Å of about 0.6×10^{-3} watts/cm² or with a two-prism spectrograph with a carbon arc source as previously described (15). The irradiance with the two-prism spectrograph was 0.17×10^{-3} watts/cm² in the region of 6100 Å, 0.24×10^{-3} watts/cm² at 7000 Å, and 0.29×10^{-3} watts/cm² at 8000 Å. The effective slit width at 7000 Å was about 200 Å. For irradiation of seedlings in the region of 4000 to 5400 Å, the spectrograph was used as a single-prism instrument, and the focal length was reduced to give a dispersion of 5 Å/mm at 5000 Å and 2 Å/mm at 4000 Å. The energies in part of this region were doubled or trebled by reduction in width of the spectrum obtained by reflection from front-surface mirrors. Combinations of neutral filters were used to give an irradiance of 0.50×10^{-3} watts/cm² with a slit width of 200 Å at 5000 Å, constant within 10 % from 4000 to 5400 Å. The absorption of the glass prisms in the spectrograph, which causes the energy to fall rapidly at wavelengths shorter than 3950 Å, limited the region studied.

Results

Course of Anthocyanin Formation: The amount of anthocyanin formed is linearly dependent upon the irradiance with the fluorescent source after 15 minutes for red cabbage seedlings and 120 minutes for turnip seedlings (fig 1). In darkness there is an endogenous synthesis of 26×10^{-10} moles anthocyanin/seedling for red cabbage. The small amount, 1.2×10^{-10} moles anthocyanin/seedling, indicated for the response in darkness of turnip seedlings, was not identified as anthocyanin. It should be considered as an arbitrary base.

Table I

Anthocyanin Formation in Turnip Seedlings as a Function of Intensity at Constant Radiant Energy from a Fluorescent Source

Light intensity	Exposure	Anthocyanin per seedling
ft-c	*hrs*	*10^{-10} moles*
1000	1	0.72
500	2	1.6
250	4	2.3
125	8	3.5

Anthocyanin synthesis in turnip seedlings depends upon the intensity at constant energy (table I). Reciprocity fails in part at least because of the long induction period.

Low Energy Response: Possible control of anthocyanin synthesis by the photomorphogenic system (4) was examined because of its response in the spectral region involved in anthocyanin synthesis. Red cabbage seedlings were exposed in some experiments for 5 minutes to radiation in the wavelength regions

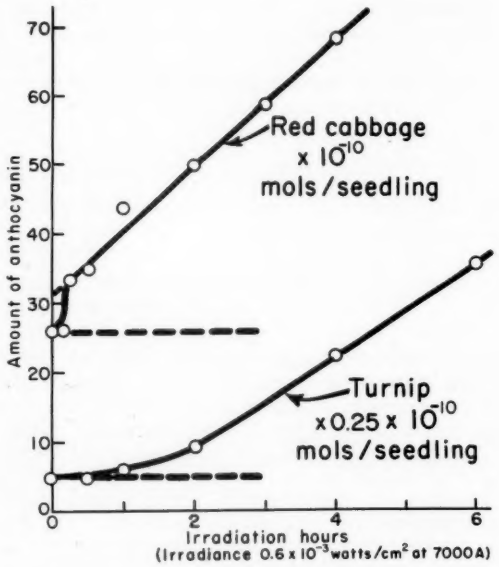

Fig. 1. Variation in anthocyanin synthesis in red cabbage and turnip seedlings with time of irradiation at a constant irradiance equivalent to photochemical effectiveness to 0.6×10^{-3} watts/cm² at 7000 Å. The seedlings were extracted for analysis 24 hrs after the beginning of irradiation. Dash lines indicate synthesis in unirradiated seedlings.

TABLE II

ANTHOCYANIN FORMATION IN TURNIP* AND RED CABBAGE SEEDLINGS IRRADIATED WITH AN ENERGY OF ABOUT 0.1 JOULE/CM² OF RED (5800 TO 6900 Å) AND/OR FAR-RED (6900 TO 8000 Å)

TYPE OF IRRADIATION	ANTHOCYANIN CONTENT PER SEEDLING	
	RED CABBAGE	TURNIP
	10^{-10} moles	*10^{-10} moles*
None	25	2.97
Red	38	2.94
Far-red	26	2.97
Far-red, red	37	...
Red, far-red	29	...

* The turnip seedlings were irradiated after induction of anthocyanin synthesis by exposure for 4 hrs to a fluorescent source.

greater than 6900 Å (far-red), which transforms the photomorphogenic pigment to the red-absorbing form. In other experiments the photomorphogenic pigment was transformed in 5 minutes to the far-red-absorbing form by exposure to radiation between 5800 to 6900 Å (red). The irradiances in both cases were about 0.30×10^{-3} watts/cm². An increase in anthocyanin synthesis as compared with synthesis in darkness was found only after exposure to red radiation (table II). This effect of red radiation of low energy on anthocyanin synthesis by the red cabbage seedlings is reversed by far-red radiation.

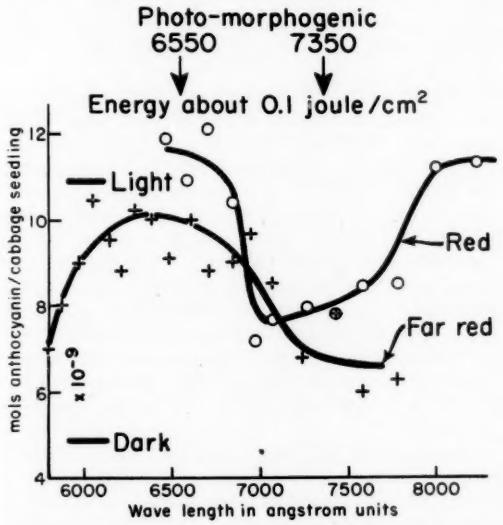

FIG. 2. Action spectra for low-energy anthocyanin synthesis by red cabbage seedlings. The curves marked red and far-red, respectively, were for lots given radiation of these types for 5 min potentiating anthocyanin synthesis as indicated by lines for light and dark immediately prior to 5-min exposures in the spectrum.

Anthocyanin synthesis in the turnip seedlings was unresponsive to the state of the photomorphogenic pigment. Turnip seedlings were irradiated for 4 hours with the fluorescent source to induce some anthocyanin formation. They were then irradiated for short times with red or far-red radiation, both of which were without effect on the synthesis (table II).

The action spectra for the low-energy anthocyanin synthesis in red cabbage seedlings were measured in the region of 5800 to 8300 Å (fig 2). Seedlings were first exposed to about 0.1 joule/cm² of either red or far-red radiation as indicated for the respective curves on figure 2. They were then placed in the spectrum for 5 minutes (radiant energy at 7000 Å of about 0.1 joule/cm²), after which they were returned to darkness for 24 hours before extraction of the anthocyanin. Anthocyanin formation in the seedlings given the prior exposure to far-red radiation is enhanced by red radiation; such enhancement accounts for the initial increase shown for red cabbage in figure 1. The synthesis in seedlings given the prior exposure to red radiation is suppressed by far-red radiation (fig 2).

HIGH ENERGY ACTION SPECTRA: The formation of anthocyanin in response to high radiant energy was measured. Dark-grown cabbage seedlings were placed along the spectrum in the region of 5500 to 9000 Å and irradiated for 4 hours. They were then irradiated with the red source for 5 minutes to convert the photomorphogenic pigment to the far-red-absorbing form (4) and then returned to darkness for 24 hours prior to extraction of anthocyanin (fig 3). The energies indicated in figure 3 were for the region of 6900 to 7100 Å. The indicated amounts of anthocyanin formed (fig 3) were those found in a single ex-

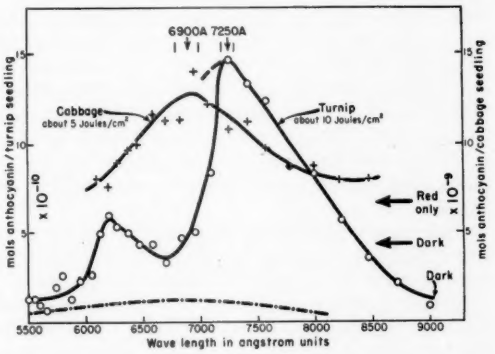

FIG. 3. Action spectra in the region 5500 to 9000 Å for anthocyanin synthesis in red cabbage and turnip seedlings. The dash line is the expected direction of energy absorption for turnip seedlings in the absence of energy transfer. The dot-dash-curve shows the relative absorption of oxidized butyryl coenzyme A dehydrogenase after Mahler (12). The heavy arrows indicate anthocyanin synthesis for darkness and a short exposure to red light. The synthesis of anthocyanin in the dark by the turnip is indicated.

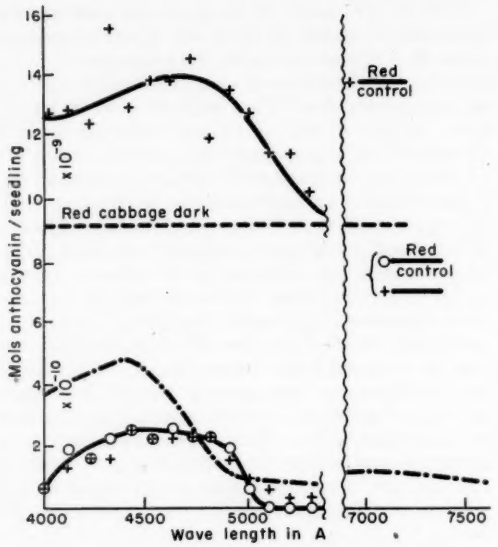

FIG. 4. Action spectra in the region of 4000 to 5330 Å for anthocyanin synthesis in red cabbage (upper) and turnip seedlings (lower) for radiant energies of about 5 and 8 joules/cm², respectively. The values in the region of 7000 Å are for control stations at the same energies with a broad slit width (400 Å). The dot-dash-curve shows the relative absorption of oxidized butyryl Co A dehydrogenase after Mahler (12).

periment. Similar results were obtained in three other experiments.

Turnip seedlings grown in darkness were first irradiated for 2 hours under the fluorescent source at 15° C to overcome the induction period shown in figure 1. They were then placed along the spectrum and exposed for 8 hours, after which they were returned to darkness for 24 hours prior to extraction of anthocyanin. Results obtained in the region of 5500 to 9000 Å are shown in figure 3. Irradiation for 2 hours at 5° C with the fluorescent source was not effective in overcoming the induction period in contrast to the effectiveness at 15° C.

The action spectra for anthocyanin synthesis by red cabbage and turnip seedlings in the region of 4000 to 5330 Å are shown in figure 4. A reference point, for synthesis in the red part of the spectrum, was run simultaneously with the same energy as for the blue part of the spectrum, but at a low resolving power with a spectral width of about 400 Å centered at 6900 Å for the red cabbage seedlings and at 7200 Å for the turnip seedlings. Duplicate samples were taken at each wavelength position and the action spectra were measured in two independent experiments for the two kinds of seedlings.

DISCUSSION

PHOTOEFFECTS ON ANTHOCYANIN SYNTHESIS: The details of the photocontrol of anthocyanin synthesis are apt to be confusing because several photoreceptive pigments are probably involved. The action maxima of these pigments is in the region of 6500 to 7400 Å. The photoreversible receptor for the photoperiodic and photomorphogenic responses of plants has an effect on anthocyanin synthesis in red cabbage seedlings but not in turnip seedlings. The two forms of this photoreversible-pigment system are indicated as red- and far-red absorbing (4). This pigment system in the far-red form probably exerts its effect on anthocyanin synthesis indirectly by influencing the metabolic status of the seedlings.

The action spectra for anthocyanin synthesis influenced by the photoreversible system (fig 2) can be separated from the other photoreactions because essential saturation is attained at low energies. Saturation for synthesis is approached at an energy of about 0.1 joule/cm² at the action maximum, while about 50 times this energy is required for an equivalent effect by the photoresponsive system next considered. The low-energy action spectra, which must be measured both for seedlings with the photoreversible pigment initially in the red-absorbing form and the far-red-absorbing form, are similar to those found for other photomorphogenic and photoperiodic responses (4).

Attention is now turned to the action spectrum (fig 3) for anthocyanin synthesis by the red cabbage seedlings in the region of linear dependence of synthesis on radiant energy (fig 1). A symmetrical curve is obtained in the region of 6000 to 8000 Å as might be expected for a single photoreceptive pigment (fig 3). The action maximum is near 6900 Å, which could have been confounded with that of the red-absorbing form of the low-energy-requiring photoreversible system (6500 Å).

The nature of the induction period for anthocyanin synthesis by the turnip seedlings is now considered. A steady state is established (fig 1) after about 2 hours with an irradiance of 0.6×10^{-3} watts/cm² in the region of 7000 Å. This induction requires radiant energy and thus involves a photoreceptive pigment. An action spectrum was not obtained for the induction period. The induction photoreaction very likely gives rise to products required as substrates for reactions preceding the steady-state photoreaction. Dark reactions are also present in the sequence of reactions between the induction and the steady-state photoreactions as indicated by the marked effect of reducing the temperature during the induction period.

Anthocyanin synthesis in turnip seedlings is most simply approached by considering the photoreactions involved in the steady-state condition (the linear portion of the lower curve, fig 1). The action spectrum in the region between 5500 to 9000 Å is shown in figure 3. A pronounced maximum is observed near 7250 Å, with a very sharp decrease in action towards shorter wavelengths. The action of a single photoreceptive pigment with a maximum near 7250 Å might have followed the direction of the dashed line

shown in figure 3 or perhaps one with a maximum effectiveness at a wavelength shorter than 7250 Å. There is evidently a "bite" being taken out of the action of the single rate-limiting photoreceptor. This "bite" is a reflection of the action spectrum of the red cabbage seedlings. This indicates that energy is probably transferred in the turnip seedlings from the receptor with the indicated maximum at 7250 Å to one with a maximum near 6900 Å, which alone is operative in the red cabbage seedlings. The energy transfer is apparently most effective from 6200 to 7200 Å. Between 6000 to 6200 Å a small action maximum is observed.

The probable energy transfer between two photoreceptors in the turnip seedlings after steady-state conditions are established tells much about the nature of these photoreceptors. First, it is unusual to find the receptor with the absorption maximum at the longer wavelength (7250 Å) transferring energy to the one with the maximum at a shorter wavelength (6900 Å). Actually, all that is required in such a transfer is for the absorption from the 7250 Å acceptor to correspond in energy to a transition from a lower energy level that is partially filled for the 6900 Å receptor. The rapid decrease with energy in the distribution for the filling of the energy levels of the ground state of the 6900 Å receptor can cause the rapid rise of the action in the turnip seedlings between 6900 to 7250 Å.

The second feature of the energy transfer in the turnip seedlings is that the photoreceptor (7250 Å) has a long half-life as indicated by the fact that it holds its excitation until the energy transfer can take place. According to Pringsheim's (17) rule on fluorescence, this indicates that the photoreceptor (7250 Å) has a conjugate double-bond system involved in the effective transition as is also probably the case in the other photoreceptor (6900 Å).

A third feature of the energy transfer in the turnip seedlings is that the two photoreceptors must be contiguous, since the energy transfer is surely not second order in kinetics.

Both photoreceptors probably contain copper, which is a reasonable expectation from the region of action. This expectation is also in harmony with the considerable inhibiting effects obtained by the use of copper chelating compounds by Edmondson and Thimann (9) on anthocyanin formation in *Spirodela oligorrhiza*. It is further indicated by reduced anthocyanin synthesis in red cabbage plants grown on copper-deficient muck found by J. C. Brown, of Plant Industry Station, Beltsville, Maryland (unpublished work).

In considering possible types of photoreceptors having the required characteristics, attention was directed to the recently described acyl dehydrogenases (13). These enzymes are flavoproteins and in the case of butyryl coenzyme A dehydrogenase, the green enzyme from liver, Mahler (12) has given evidence for associated copper. The butyryl coenzyme A dehydrogenase has an absorption maximum in the region of 6800 to 6850 Å (12) for the oxidized form, which is in agreement with that for the red cabbage photoreceptor (6900 Å). Recently, Steyn-Parve and Beinert (16) have raised questions about the necessity of copper for the functioning of the enzyme. They suggest that absorption in the red region of the spectrum is present for the copper-free enzyme.

Irrespective of the exact nature of the acyl dehydrogenases other than as flavoproteins, their consideration suggests that the photoreceptive pigments for anthocyanin might be effective in the blue portion of the spectrum, where the flavin moiety absorbs. Attention was accordingly given to the nature of the action spectra in the region of 4000 to 5000 Å, which had not earlier been measured because of the experimental difficulties in obtaining the requisite irradiances. To attain this end the spectrograph was modified to a single-prism instrument of shorter focal length and otherwise changed as previously mentioned. The action spectra were then measured (fig 4). The results are in agreement with action arising from a flavin absorption and are evidence for the flavoprotein nature of the photoreceptors.

The action (amount of anthocyanin formed) per incident erg or per incident quantum is greater in the region of the red maxima than between 4000 to 5000 Å, particularly by the turnip seedlings, while the absorption coefficient of butyryl coenzyme A dehydrogenase enzyme is about a third as great. This indicates that the quantum efficiency is greater for absorption in the red than in the blue part of the spectrum. In the red part of the spectrum the action could be associated with the copper atom. Mahler (12) showed by poisoning with cyanide that the copper atoms are probably required for single-electron transport.

The photoreactions are possibly concerned with electron or hydrogen transfer in the formation of the aromatic A- or B-ring of the anthocyanin molecules or on precursors of these rings such as acyl radicals. (See Geissman and Hinreiner (11) for the most recent discussion of anthocyanin biosynthesis.) Since the probable photoreceptor is the oxidized form of the flavoprotein, the photoactions are effective in its return to the reduced form.

The effects of light on anthocyanin synthesis by cress seedlings noted by Eddy and Mapson (8) were probably due to the influence of the reversible pigment system that produces photomorphogenic and photoperiodic responses as indicated by the effectiveness of the low radiant energies used. Effectiveness of radiation in the region of 7250 Å for inducing anthocyanin formation in bean seedlings was noted by Withrow, Klein, Price, and Elstad (21). They commented that this synthesis paralleled the photomorphogenic response although they did not consider their experiments complete in this respect. The observed enhancement of anthocyanin synthesis at 7250 Å in the bean seedlings was more likely due to a photoreceptor similar to the one found in turnip seedlings with a maximum at 7250 Å. The irradiance

(1500 microwatts/cm²—7250 Å treatment) in the experiments on anthocyanin formation in the bean seedlings was sufficient for promoting synthesis by such a photoreceptor. Also, anthocyanin production was energy-dependent for high energies.

QUENCHING OF PHOTOSYNTHESIS: The probable energy transfer between photoreceptors involved in anthocyanin synthesis suggests an explanation for the rapid decrease in photosynthesis in Chlorella in the region of 6750 to 7000 Å as observed by Emerson and Lewis (10) and verified by others. The speculation here is that a flavoprotein with an absorption maximum near 6900 Å in the plastid quenches energy transfer in the chlorophyll-carotinoid coupled system of the plastid. This flavoprotein could be one associated with electron and hydrogen transfer resulting in oxygen evolution and transferring of hydrogen from water (6). The implication should not be made that the system for anthocyanin synthesis is associated with the plastid.

SUMMARY

Action spectra were measured for anthocyanin synthesis in 3-day-old seedlings of Red Acre red cabbage, *Brassica oleracea*, and Purple Top White Globe turnip, *Brassica rapa*. A single photoreceptive pigment is present in the red cabbage seedlings leading to action maxima at 6900 Å and near 4500 Å. The receptor is possibly a flavoprotein similar to the butyryl coenzyme A dehydrogenase enzyme of liver mitochondria, and it possibly contains copper. Action maxima for anthocyanin synthesis in turnip seedlings were found near 7250 Å, 6200 Å, and 4500 Å. The action spectrum in the region of 6200 to 7200 Å suggests that energy of activation is transferred between two photoreceptors. A speculation is advanced that a similar quenching of photosynthesis takes place in the plastid in the region of 6750 to 7000 Å, accounting for the decreased action in this region as observed by others.

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TRANSLOCATION OF ORGANIC SUBSTANCES IN TREES. II. ON THE TRANSLOCATION MECHANISM IN THE PHLOEM OF WHITE ASH (FRAXINUS AMERICANA L.)[1]

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The mechanism of phloem translocation is a major problem. A simple and plausible theory, the mass flow theory, has been proposed by Münch in 1930 (17). The driving force presumably is a difference in turgor pressures. In the leaves, where organic material is synthesized, the osmotic pressure is high. In stem and roots, where assimilates are removed for growth and storage, the osmotic pressure is low. According to Münch, the resulting turgor pressure difference automatically forces vacuolar sap from places of production to places of consumption. The alternative theories are less clearly outlined. They differ from the mass flow theory in supposing that the organic molecules are metabolically or physically translocated, but do not move as a solution in bulk.

Much evidence for and against mass flow has been presented since 1930, but no final decision can yet be made, because most of the disagreements rest upon interpretation of the data. However, if mass flow does occur, it seems to be restricted to the sieve elements of the phloem. Translocation from the parenchyma cells of the leaves into the sieve elements may occur against a concentration gradient (19) and chemical transformations are considerably involved (10, 25, 27). Removal of sugars from the sieve tubes also seems to be a metabolic process (27, 29). Chemical constituents within the sieve tube system, however, were found to be qualitatively the same throughout the plant (10, 16, 26, 27). These chemical findings are not surprising because anatomists observed many years ago that the metabolic activity of sieve elements must be reduced: their protoplast lacks a nucleus when the cell completes its development and becomes functional (5).

There are three basic requirements for a passive movement of solutes along a turgor pressure gradient:

1st. The cytoplasm lining the longitudinal walls of the sieve tubes must be semipermeable.

2nd. The sieve plates, however, must be permeable to the translocated solution.

3rd. The turgor pressure gradient must be positive in the direction of flow.

Some advocates of Münch's theory believe today that a passive mass flow through the sieve elements is indirectly maintained by the metabolic activity of the sieve tube cytoplasm and the surrounding tissue, particularly the companion cells. In the leaves, assimilates are secreted into the vacuole of the sieve elements, in stem and roots they are metabolically removed. Thus a concentration gradient is established and maintained.

Although the turgor gradient has to be positive in the direction of flow, according to the mass flow theory, the turgor itself does not necessarily have to be positive. Mass flow may be "suction"-flow as well as "pressure"-flow. This has extensively been investigated by Frey-Wyssling (6). Metabolic removal of organic material from the sieve elements (e.g., through starch formation) must be followed by a loss of a corresponding amount of water. This results in a turgor drop and, if requirements one and two are fulfilled, in a flow of vacuolar sap toward the point of removal of organic material.

In the experiments here reported an attempt has been made to investigate the three conditions necessary for mass flow to occur, using white ash as experimental material. There are several advantages of studying phloem translocation by means of chromatographic analysis of sieve tube exudate of trees. Since it is quite likely that various chemical reactions account for entry of solutes into and exit from sieve elements, and since translocation from the parenchyma cells in the leaves into the sieve tubes occurs against a concentration gradient, osmotic pressure determinations of whole organs or even vascular bundles are no indication of the osmotic pressure within the sieve tubes. Evidence indicating that sieve tube exudate of trees represents translocated material has been previously discussed (28). Crafts and Lorenz (3) stated that "phloem exudate from cucurbits can no longer be considered as a true sample of the assimilate stream." This statement is based upon the observation that nitrogen content of phloem exudate from cucurbits is much higher than the nitrogen content of their fruits. This does not hold, however, for trees. Nitrogen content in exudate of trees is very low indeed (16, 27, 29). Furthermore, exudation from one tapping cut lasts a considerable length of time in many trees, in white ash for instance, for half an hour or longer. The material, therefore, originates from the sieve tubes far above the cut. This is also indicated by the observation of Hartig (7, 8) and Münch (17) that the sieve tube turgor is reduced up to several meters above the tapping cut.

The situation in a large forest tree is a much clearer one than that in a small herbaceous plant. In order to investigate gradients, we can take samples of sieve tube exudate from places on the trunk which are far apart but nevertheless physiologically comparable, and clearly separated from the place of assimilate production. Moreover, we do not have to cut the entire conducting system like in cucurbits, but disturb only a very small fraction of the whole phloem with each cut.

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The total molar concentration gradient is undoubtedly a very close measure of the turgor gradient, it is therefore relatively easy to investigate the third requirement. It is much more difficult, however, to investigate the properties of the sieve tube cytoplasm. One approach is the plasmolysis experiment. Mature sieve tubes are difficult to plasmolyze and great skill is needed for this operation (4, 20), but this difficulty may be due only to their longitudinal permeability. Thus application of a solution of high osmotic value may cause a flow of vacuolar sap through the sieve tubes toward the point of application, rather than plasmolysis of individual sieve elements. The interpretation of plasmolysis experiments is still being debated (1, 4, 21). In the experiments here reported an attempt has been made to approach the problem of semipermeability in a different way.

Experimental

All experiments were carried out on trees in the Prospect Hill tracts I, II and IX of the Harvard Forest. Sieve tube evudate was obtained from white ash by cutting into the bark as previously described (28). The samples were immediately taken up with numbered pipettes, calibrated at 1 (for mannitol analysis), 4 (sugars) or 5 (sugars, amino-acids) lambdas and transferred to the starting point of paper chromatograms. As soon as these were dry they were wrapped in polyethylene foil and placed in a freezer at $-20°$ C. Each package contained the analytical work for one day, i.e., 56 sugar analyses including 16 reference values of known amounts.

The identification of the oligosaccharides (sucrose, raffinose and stachyose) in the sieve tube exudate of white ash has been previously described (28). Whatman No. 1 paper was used in a descending procedure throughout. The partitioning solvent was *n*-butanol : acetic-acid : water = 3 : 3 : 2 v/v, which yields even separation of sucrose, raffinose and stachyose within 24 to 30 hours. Each chromatogram contained five exudate samples, three for localization of the sugar positions and two for analysis. After separation the paper was cut into five strips and the three reference strips developed with a resorcinol reagent (28) for localization of the sugar positions. The elution process is shown in figure 1. Each sugar is cut out and eluted from the paper into test tubes by a chromatographic process using distilled water. The racks were welded from 1/16″ polyethylene sheet. Each rack holds 8 test tubes calibrated at 5 ml and numbered. Since the elution process must take place in a moist atmosphere, each rack was covered with a glass jar. To achieve the same blank value for every sample the short paper pieces (usually raffinose, sometimes also stachyose) were brought to the standard length by adding a piece of blank paper previously treated, like the chromatograms, with the partitioning solvent. During elution the oligosaccharides were enzymatically hydrolyzed with 10 lambdas of an invertase solution (Nutritional Biochemical Co.) placed on the tip of each paper before elution. This

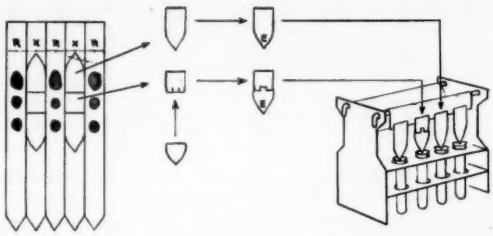


Fig. 1. Elution and hydrolysis of sugars. The chromatogram is cut into five strips, of which three reference strips (R) are developed for localization of sugar positions. Sugars to be analyzed (on strips X) are cut out, the short pieces containing raffinose brought to standard length by adding a piece of blank paper. Ten lambdas of invertase is placed on tip of each paper (E). Papers containing sugars are attached to filter paper whose opposite end dips into the water in the trough of elution rack. To keep the atmosphere moist during elution, the racks are covered with glass jars. The sugars are eluted for 5 hours in the presence of toluene (to prevent infections). At the same time they are hydrolyzed (fructose unit split off) by the enzyme.

enzyme splits off the fructose unit of each sugar. Special attention was necessary to prevent infection during the elution and hydrolysis process. It is practically impossible to keep the set-up completely sterile. Antisiphon rods, anchor rods and filter paper were heated and the polyethylene racks exposed to strong ultraviolet light before use. Toluene was placed in the trough of the elution rack and a few drops introduced into each test tube. Elution and hydrolysis were complete after 5 hours. In order to avoid losses by infection, no longer time should be used. After elution the volume in each test tube was brought to 5 ml with distilled water and the sugar determination carried out with Somogyi's method (22), as modified by Nelson (18). The reaction of sucrose and raffinose was the same for same molar amounts, that is, the reaction intensity of glucose and melibiose were the same under our conditions. The calibration curve obtained from sucrose and raffinose reference values could also be used for stachyose determinations, because the fructose molarity of inverted exudate samples was found to be the same as the total molarity of the three sugars determined with the described procedure.

The quantitative determination of mannitol was carried out by transmission densitometry after the chromatograms had been developed with an alkaline silver nitrate reagent (24). Because transmission densitometry is much less accurate than chemical methods, a large number of samples have been analyzed for each needed value.

Semipermeability of the Sieve Tube Cytoplasm: The physiological properties of the sieve tubes required by the mass flow theory of Münch (17) are rather peculiar. The whole sieve tube system must behave somewhat like a single giant cell. The con-

centration of its contents has been shown to be 10 to 30 grams per 100 ml of sap (7, 8, 13, 17, 27). About 90 % of the solutes are sugars, either sucrose alone (13, 26, 27, 28), or accompanied by higher oligosaccharides (28). Sieve tube exudate of ash contains also a large amount of mannitol (up to 0.2 molar in white ash). The high concentration of the sieve tube or sieve cell sap produces a corresponding turgor within the sieve tube system which causes an elastic expansion of the cells. When the system is severed by a tapping cut, some sap is forced out within a few seconds by the elasticity of the cell walls. Many trees, e.g., conifers, cannot be tapped, however. This has been claimed to be evidence against mass flow. However, current investigations by Mittler (14, 15, 16) with cut-off stylets of aphids seem to promise that exudate can be obtained from any plant with functional sieve elements. In most trees tapping causes an explosion-like exudation due to elastic contraction of the sieve tubes. The flow then stops, probably because of a plugging of the sieve plates at the moment of the pressure release (2, 12). In other trees, such as white ash, flow continues slowly for about half an hour or longer.

As soon as the pressure is released by tapping, water enters the system from the surrounding tissue thus diluting the exudate. This dilution, which begins at the moment the cut is made, can be measured. Ash trees about 5 cm in diameter were tapped by a 2-cm long cut. Samples were taken with numbered pipettes graduated at 5 lambdas. After the first sample had been taken the excess sap, exuded because of the immediate elastic contraction of the sieve tubes, was usually blotted from the cut. Subsequent samples were taken as soon as possible. During the month of August, 6 to 12 samples could be obtained within about half an hour. (From about mid-September on the sap flow greatly increases, its concentration decreases and the dilution effect decreases. This indicates a greater longitudinal permeability of the sieve tubes. Seasonal changes have been discussed elsewhere (29)). It was necessary to work on calm days to avoid an increase in concentration caused by drying of the sap on the cut surface. Moreover, the cut was carefully shielded with Scotch tape and only a small opening left for the pipettes. The sugar concentration of the exudate decreased continuously. Figure 2 shows an experiment carried out on August 20, 1956. In the last sample, taken 25 minutes after the cut was made, the concentration had dropped to about 70 % of that in the first one. The same dilution effect was observed by Frey-Wyssling in the latex of Hevea (6). The results of Crafts (3) and Tingley (23) show a similar trend in the phloem of cucurbits, but since the exudation in cucurbits is small, and less subtile methods were available in 1944, the dilution was found through a series of cuts.

Further evidence for the semipermeability of the cytoplasm lining the side walls of the sieve tubes is the following observation. The sieve tube system does not leak; it retains the sugars even for a day or

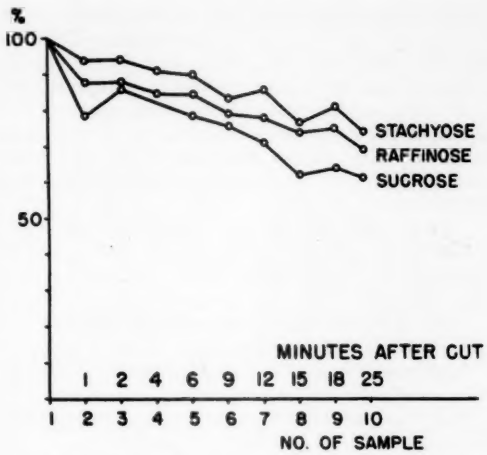


FIG. 2. Osmotic dilution effect in the sieve tube system of white ash (Aug. 20, 1956). Subsequent samples taken from one tapping cut show a continuous decrease in concentration. The cut yielded sap for about half an hour. The sugar concentrations are shown in percent of the concentrations in the first sample which are: stachyose 0.242 M, raffinose 0.077 M, sucrose 0.068 M.

two after all the leaves have dropped in the fall (cf fig 4). During this time the sugar concentration decreases continuously by the activity of an enzymatic removal system (29).

The gradients also suggest that the side wall cytoplasm of the sieve tubes is semipermeable. If the sieve tubes were leaking the gradients had to correspond with the molecular sizes. In other words, the smallest molecule, mannitol, had to have the steepest gradient, the largest molecule, stachyose, the gentlest. However, the gradients have never been found to be correlated with the molecular size. The molecules must be metabolically removed from the sieve tubes.

PERMEABILITY OF THE SIEVE TUBES IN THE LONGITUDINAL DIRECTION: The flow of exudate from a tapping cut indicates that the sieve plates are permeable for the solution. After the first explosion-like exudation which is due to the release of the elastic tension of the sieve tubes (17), the flow continues for about half an hour. The dilution effect indicates that this second stage of exudation is maintained by osmosis. The question is whether the same kind of solute movement occurs under natural conditions. If the tapping process were a severe disturbance and the solute flow an artifact, the sieve tubes, and especially the sieve plates immediately above the cut would be seriously damaged and could no longer function. (The turgor is released up to several meters above the cut (17).) We found, however, that any time after tapping (from half an hour to several weeks later), the sieve tubes above the cut can be tapped again. They are not damaged by tapping. Therefore, it does not seem likely that the permeability of the sieve plates is an artifact.

CONCENTRATION GRADIENTS IN THE SIEVE TUBE SYSTEM: No matter where we tap the tree, 15 meters above the ground near the leaves, or at the base of the trunk, the content of the exudate is qualitavely the same. The concentration varies from tree to tree and fluctuates somewhat diurnally (13, 27, 29) and seasonally (29). Concentrations of the substances found in white ash were usually within the following values:

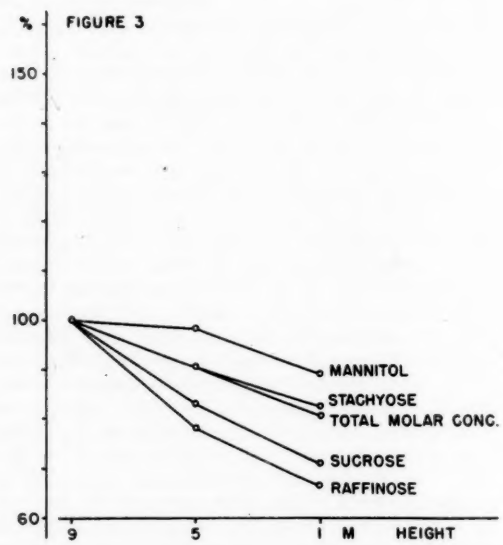
Sucrose, 0.05–0.15 M
Raffinose, 0.05–0.1 M
Stachyose, 0.15–0.3 M
Verbascose, trace
(No reducing sugars, no sugar phosphates)
Mannitol, 0.05–0.2 M

Amino-acids and amides usually less than 0.001 M total, mainly glutamine and glutamic acid.

Traces of substances absorbing or fluorescent in ultraviolet light.

Samples were taken from a tree 20 to 30 cm in diameter at 3.5-hour intervals during 24 hours at 1, 5 and 9 meters height. This was repeated with many trees during the summer months of 1955 and 1956. Although gradients showed slight diurnal variations as reported elsewhere (29), they were always positive in the downward direction of the trunk. Only one substance, fluorescent in 365 mμ when exposed to short ultraviolet light, increased in concentration in the downward direction. The concentration of this substance was measured on the chromatogram by transmission densitometry. The substance is also present in winter when there is no phloem exudation at all. One drop of water applied at this time on a fresh cut and immediately picked up again contained about as much of the fluorescent substance as a sample of sieve tube exudate obtained in summer. This suggests that it does not originate in the sieve tubes but is eluted from the bark tissue during the tapping process.

The substances present in sufficient concentrations to account for an osmotic pressure difference are sucrose, raffinose, stachyose and mannitol. Substances other than sugars and mannitol have been found to represent only about 10 % of the total dry weight. Since the concentration differences over a length of 8 meters are around 20 % the described gradients cannot be affected more than 2 % by other substances. Concentration gradients are shown in figure 3. Although all four substances decrease in concentration on their way down the trunk, they do not decrease at the same rate in every individual tree. In other trees, for instance, stachyose showed the greatest decrease, raffinose decreased less, sucrose least, and so forth. This decrease is ascribed to the removal mechanisms which have been discussed elsewhere (29).



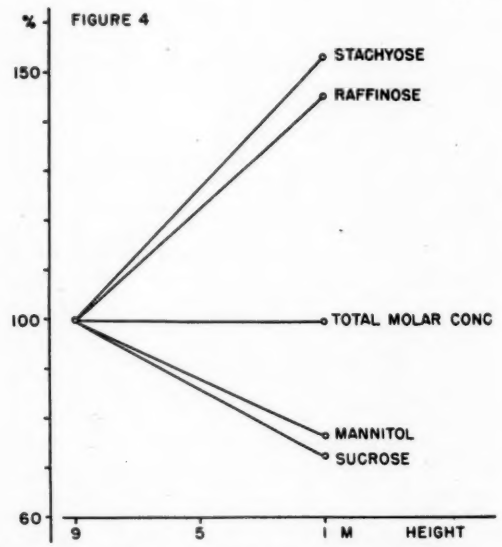


FIG. 3. Typical gradients in sieve tube exudate of white ash (July 30/31, 1956). Concentrations are shown in percent of the 9-meter values, which are: sucrose 0.082 M, raffinose 0.073 M, stachyose 0.202 M, mannitol 0.178 M. Values at each height are average values of 24 samples taken at 3.5-hour intervals during 24 hours. Concentration gradients were always positive in the downward direction of the trunk throughout the summer.

FIG. 4. Gradients of the four substances that can account for an osmotic pressure difference (Oct. 8, 1956, after all leaves have dropped). Concentrations are shown in percent of the 9-meter values, which are: sucrose 0.138 M, raffinose 0.034 M, stachyose 0.099 M, mannitol 0.136 M. Values at each height are average values of 32 samples from 16 cuts around the trunk. Some gradients are negative in the downward direction and the total molar gradient has almost disappeared. The gradient inversion begins when the leaves turn yellow. This figure shows a late stage.

Important for us at this point is the fact that the four substances do decrease at different rates, whatever is the cause. What should happen, according to the mass flow theory, at the time the leaves cease to supply material to the sieve tubes? The flow should continue as long as there is a turgor difference at the different levels (high up in the tree near the leaves, and at the base of the trunk). There will be a turgor difference as long as there is a difference in the total molar concentration in the sieve tubes. If there is only one osmotically active substance (e.g., sucrose), the flow will slow down and finally stop completely when the concentration reaches the same value everywhere. If there are several substances and their concentration gradients are different, the substances with the smallest gradients—still according to the mass flow theory—have to be moved against their gradients until the total molar gradient disappears. This, of course, is only strictly true if the total molar concentration gradient is identical with the turgor gradient. However, at this point, we are mainly interested in the change that takes place when the leaves cease to supply assimilates to the phloem.

Experimental results indicate that this actually happens in the fall. The sieve tubes can be tapped until one or two days after all the leaves have dropped. Figure 4 shows an experiment where 32 samples (from 16 cuts around the trunk) have been taken at 1 and 9 meters respectively from a tree whose leaves had all dropped. Stachyose and raffinose show a large negative, sucrose and mannitol a postive gradient. The gradient of the total molar concentration is practically zero. This gradient inversion, a typical autumn effect, starts to appear when the leaves turn yellow and begin to drop.

The results here presented are in agreement with the mass flow theory of Münch (17), according to which translocation takes place through the vacuole of the sieve tubes, a difference in the total turgor pressure being the driving force of translocation. Indeed, it would be very difficult to interpret them in terms of a metabolic transport. We do not know, however, to what extent these conclusions, drawn from experimental results with white ash are applicable to other plants. Huber found very large sieve pores (up to 15μ in diameter) in *Fraxinus excelsior* and these have no callose cylinder (9, 10, 11). However, many other plants have rather small pores usually filled with cytoplasm. The fact that investigators are not in agreement on the translocation mechanism may not be due only to individual interpretation of results, but also to the variety of plants used for experimental work, and it would not be surprising if there were more than one single mechanism that can account for the translocation through the phloem.

Summary

Sieve tube exudate of white ash (*Fraxinus americana* L.) has been analyzed. The same substances are found in all parts of the tree. Their concentrations vary somewhat from tree to tree: Sucrose 0.05 to 0.15 M; raffinose 0.05 to 0.1 M; stachyose 0.15 to 0.3 M; a trace verbascose; mannitol 0.05 to 0.2 M; amino-acids and amides usually less than 0.001 M, mainly glutamine and glutamic acid; traces of substances that are absorbing or fluorescent in the ultraviolet light. No reducing sugars and no sugar phosphates have been found.

There are three basic requirements for Münch's mass flow mechanism through the sieve tubes: 1) The cytoplasm lining the longitudinal walls of the sieve tubes must be semipermeable. 2) The sieve plates must be permeable for the transported solution. 3) The turgor gradient must be positive in the direction of flow. Experimental results with white ash are all in agreement with these requirements:

1. Three observations indicate the semipermeability of the side wall cytoplasm of the sieve tubes: (a) A series of samples taken from one tapping cut show a gradual decrease in concentration to a final value of about 70 % of that of the first sample. This is ascribed to an osmotic dilution with water from the surrounding tissue after the release of the sieve tube turgor. (b) The turgor of the sieve tubes is retained for one or two days after all the leaves have dropped in the fall. (c) There is no correlation between molecular size and gradient (no steeper gradients of substances with smaller molecules) which would suggest leaking of the sieve tubes.

2. A mass flow obviously does occur during about half an hour after tapping; the sieve plates are therefore permeable during this time. The sieve tubes immediately above the cut can be tapped by a second cut at any time after application of the first cut. This observation suggests that the permeability of the sieve plates is not an artifact.

3. The total molar concentration, undoubtedly a close measure of the turgor pressure gradient, is positive in the downward direction of the trunk throughout the summer. The gradients of the different substances are not the same, however. Therefore, in the fall, when the leaves form their abscission layer and cease supplying assimilates to the phloem, a gradient inversion of some of the substances takes place, and the total molar gradient disappears.

In agreement with other workers, the presented data suggest that there is a passive flow of solutes along a positive turgor gradient which is established and maintained by the metabolic entry of assimilates into and removal from the sieve tubes.

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THE EFFECT OF SILICON ON YIELD AND MANGANESE-54 UPTAKE AND DISTRIBUTION IN THE LEAVES OF BARLEY PLANTS GROWN IN CULTURE SOLUTIONS[1]

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Silicon in the form of its compounds occupies 87 % of the earth's crust and constitutes a large percentage of the ash of some plants grown in soil. The addition of silicon to culture solutions has been shown by a number of workers (2, 3, 5) to result in an increase in plant yield. In spite of its beneficial character, and the large amounts absorbed by plants, this element has not been shown to be essential to higher plants in the strict sense of the word. The beneficial effects obtained by the addition of silicon to culture solutions may well be due to secondary causes rather than to an essentiality on the part of silicon.

Previous work in this department by A. Ulrich (unpublished data) concerning the effect of silicon on yield and on the necrotic symptoms observed on the leaves of barley plants grown in culture solutions,

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would also seem to suggest that silicon is an essential element. Ulrich found that the appearance of the dark brown spots observed on leaves of barley plants could be delayed by the addition of soluble silicates to the culture solution with a resulting marked increase in yield. This effect was also observed by the authors (6, 7) in Hoagland culture solutions in which the macro-salt was maintained at both one-fifth and full strength concentrations. That this effect was not peculiar to silicon was shown when the necrosis was eliminated by increasing the macro-salt to full or double strength or by decreasing the micro-salt (particularly Mn) concentrations of the solutions.

Experimental

The culture solutions used in the following experiments were, with one exception (footnote table II), that of 20 % Hoagland Solution, #2. The salts used consisted of CP or reagent grade chemicals dissolved in distilled water. The resulting solutions, adjusted to pH 5.5 with $Ca(OH_2)$ or H_2SO_4, were kept well aerated. The following table gives the salts and concentrations used in preparing the 20 % solutions. The full strength solution was prepared by increasing the macro-salt concentrations to 5 times the value given while keeping the micro-salt concentrations as shown.

Macro-salt	Concentration, millimoles	Micro-salt	Concentration,* ppm
$NH_4H_2PO_4$	0.2	H_3BO_3	0.025
KNO_3	1.2	$MnCl_2$	0.5 or 5.0
$Ca(NO_3)_2$	0.8	$ZnSO_4$	0.05
$MgSO_4$	0.4	$CuSO_4$	0.02
		H_2MoO_4	0.01
		$FeSO_4$**	0.10

* Concentration refers to the element italicized in the micro-salt.

** Fe was added twice weekly to each solution at 0.10 ppm.

An experiment was conducted from April 29 to June 10, 1954 to determine what effect the addition of silicon to a 20 % Hoagland solution #2 (1) would have upon the growth of Atlas Barley (*Hordeum vulgare* L.). Five one-week-old barley seedlings were placed in 45 liters of culture solution containing Mn at a concentration of 0.5 ppm. The plants were grown in the presence and absence of 10 ppm of silicon which was added to the solutions as sodium silicate (adjusted to pH 5.5 with H_2SO_4). Plants were harvested at 4 and 6 weeks, oven dried at 70° C and analyzed colorimetrically for Mn following a HNO_3 digestion and the development of the $KMnO_4$ color with periodate in the presence of phosphoric acid. The concentration of Mn found in the plants as well as yield data are shown in table I.

A second experiment was carried out during the period November 10 to December 20, 1955 in which individual barley seedlings were placed in Pyrex beakers containing 2 liters of one-fifth strength Hoagland solution. The initial Mn concentration in all beakers was 5.0 ppm and silicon as sodium silicate was added to one-half of the solutions at a concentration of 30 ppm. The plants were harvested at 6 weeks, oven dried, and weighed. Yield data for this experiment is given in table II.

Table I

The Effect of Silicon on the Manganese Content of Leaves and on the Yields of Barley Plants Grown in 20 % Hoagland Solution with 0.5 ppm Mn

Treatment	Yield (gm plant)		Mn in plant (ppm)			Necrosis in old leaves
	Top	Root	New leaves	Old leaves	Tops	
			A. 4-wks growth			
+Si	4.0	2.0	61	303	171	None
−Si	1.6	0.9	104	305	204	Moderate
			B. 6-wks growth			
+Si	14.4	7.2	53	412	192	None
−Si	11.2	3.8	37	410	184	Severe

It is evident from the data in tables I and II that the addition of silicon had a marked beneficial effect on both root and top growth. This increase in yield is in agreement with the data reported by other workers (2, 3, 5). A probable reason for the increase in yield shown here may be ascertained from the last columns of tables I and II. These columns show the amount of necrosis, reported as visible symptoms of Mn toxicity as observed on the plants. Necrotic symptoms were entirely absent in plants grown in culture solutions containing silicon whereas very heavy spotting was observed at 6 weeks in plants grown in the absence of silicon. This necrosis, occurring on all of the older leaves, effectively reduced the photosynthetic capacity of the plants as compared with the healthy green plants grown in the presence of silicon. An increase in yield with a decrease in necrosis was

Table II

The Effect of Silicon on Yield and Necrotic Symptoms of Barley Grown in 20 % Hoagland Solution with 5 ppm Mn

	Yield of tops gms/plant *		Yield of roots gms/plant		Mn toxicity symptoms	
	+Si	−Si	+Si	−Si	+Si	−Si
	1.41	0.46	0.43	0.13	None	Heavy spotting
	1.31	0.52	0.38	0.13	None	"
	1.46	0.51	0.52	0.14	None	"
	1.56	0.53	0.45	0.16	None	"
	1.51	0.61	0.41	0.16	None	"
Av	1.45	0.53	0.44	0.14		

* Plants grown in full strength Hoagland solution containing 5 ppm Mn in the absence of silicon had a top wt of 1.24 gm, a root wt of 0.3 gm, and were slightly spotted.

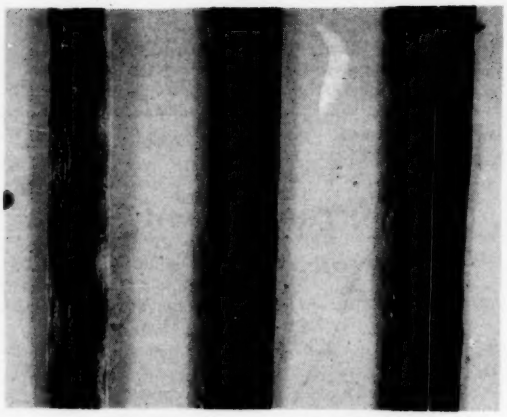

FIG. 1. Photograph of the 4th, 5th, and 6th leaves of barley plants grown in Hoagland solution in the presence of 5.0 ppm Mn and 30.0 ppm Si.

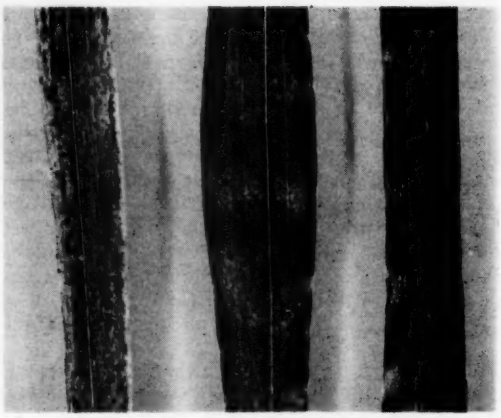

FIG. 3. Photograph of the 4th, 5th, and 6th leaves of barley plants grown in Hoagland solution in the presence of 5.0 ppm Mn and in the absence of Si.

also obtained by increasing the macro-salt concentration from one-fifth to full Hoagland solution (table II). A top weight of 1.24 gm and a root weight of 0.3 gm was obtained with full strength Hoagland solution in the absence of silicon. These values are nearly as great as the 1.45 gm tops and 0.5 gm roots obtained with one-fifth strength solutions in the presence of silicon and are considerably greater than the 0.44 gm tops and 0.14 gm roots obtained with one-fifth strength solutions in the absence of silicon. The barley plants in the full strength Hoagland solutions were only slightly spotted while those in the one-fifth strength solutions were heavily spotted when silicon was absent. Thus, the higher macro-salt concentration as well as the addition of silicon reduced necrosis and produced a greater yield than was obtained for plants grown in one-fifth strength solutions in the absence of silicon.

Although silicon had a marked effect on yield it is clear from table I that it had no apparent effect on the Mn content of the old leaf tissue. The concentration of Mn in the leaves at 4 weeks was 300 ppm and at 6 weeks 400 ppm regardless of whether silicon had been added to the culture solutions or not. The Mn content of the roots and new leaves were quite variable. The similarity in the Mn content of old leaves in the presence or absence of silicon is markedly different from the effect exhibited by an increase in the macro-salt concentration of the solution. In the latter case the added salt is antagonistic to Mn and represses the uptake of Mn by the plant (7) thus decreasing the Mn concentration in the leaves. If the necrotic spots observed in the absence of silicon are a result of an increase in the Mn content of the tissue to a toxic level, then the plants grown in the presence of silicon, having the same level of Mn in the older leaves might have a different distribution of Mn within the leaves.

To investigate this possibility an experiment was carried out using radioactive Mn^{54}. The experimental

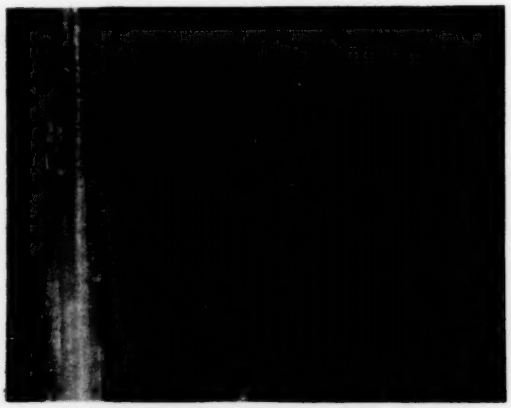

FIG. 2. Corresponding radioautograph of leaves shown in figure 1.

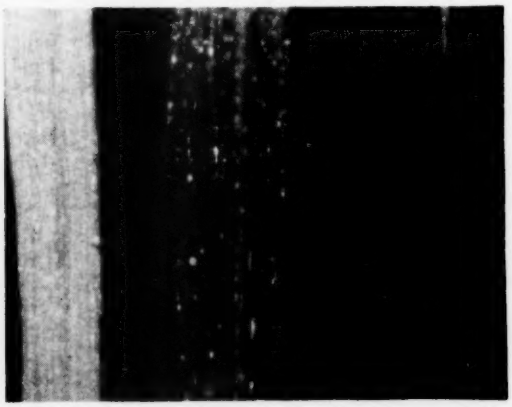

FIG. 4. Corresponding radioautograph of leaves shown in figure 3.

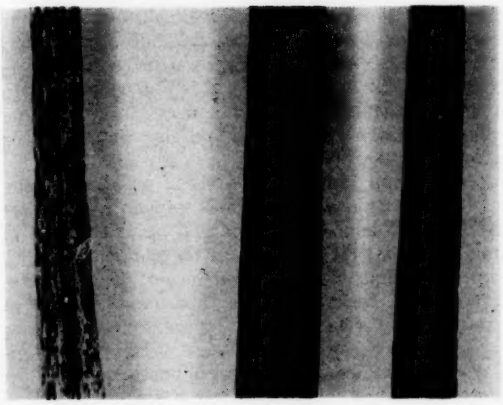

FIG. 5. Photograph of barley leaves showing 3 stages of necrosis.

set up was the same as that described above for the second experiment with Mn at 5.0 ppm and Si at 30 ppm. Twelve microcuries of Mn^{54} were added to each of 4 beakers at the beginning of the experiment and to an equal number of beakers following three weeks of growth. In each instance two of the beakers contained 30 ppm Si and 2 of the beakers contained no Si. The plants were harvested at 6 weeks and the 4th, 5th, and 6th leaves of plants which had been growing in the presence of Mn^{54} from the beginning of the experiment were selected for radioautographs. These leaves were chosen in order to examine autographs of heavily, moderately, and lightly spotted leaves. The leaves were pressed between blotters and oven dried at 70° C prior to exposure to film. Enlarged sections of the radioautographs are shown in figures 2, 4, and 6. Photographs of the same leaf areas are shown in figures 1, 3, and 5.

A distinct difference is observed in the distribution of Mn in the leaf tissue of the silicon and non-silicon treatments (figs 2 and 4). The leaves from the silicon treatment show a decidedly more even distribution of Mn^{54} than is observed in the leaves of the non-silicon treatment. In both cases an increase in the intensity of radioactivity is observed in going from the youngest to the oldest leaf. Radioactive islands or spots are observed in the same location on the leaf autograph as are the necrotic spots on the photographs (figs 1 and 3). This fact is most noticeable in the moderately spotted, center, leaf in figures 3 and 4. Both the radioautograph (fig 2) and the photograph (fig 1) of leaves from the silicon treatment show a complete absence of spotting due to radioactive islands or necrotic tissue. This absence of localized areas of concentrated radioactivity shows the unique effect of silicon in changing the Mn distribution in the plant tissue so that a toxic quantity does not accumulate as is found in its absence. A comparison of the youngest leaf from the non-silicon treatment in figures 5 and 6 shows the presence of radioactive islands where no visible necroses have developed. It would thus appear that the Mn concentration while higher in these areas has not yet increased to a toxic level resulting in visible injury to the tissue. A greater quantity of radioactive islands in the absence of necrosis might have been observed if the ratio of Mn^{54} to total Mn had been greater.

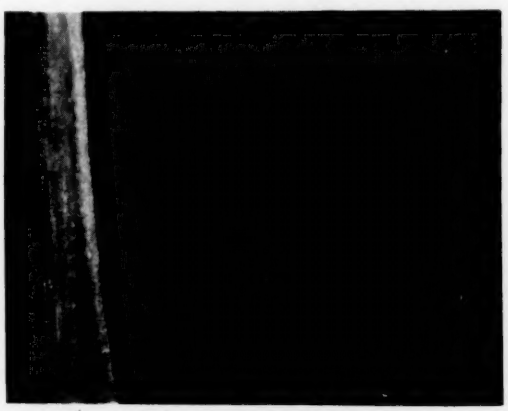

FIG. 6. Radioautograph of leaves from figure 5 showing radioactivity in areas where no visible spotting has appeared as yet.

TABLE III

DISTRIBUTION OF Mn^{54} IN BARLEY LEAVES WHEN ADDED AT THE BEGINNING OR THE THIRD WEEK OF EXPERIMENT

Si	Cotyledon	Leaves		
		1st	2nd	3rd
	A. Mn^{54} added at beginning of the expt			
+	.... *	3790	2260	2120
+	....	3640	2720	2380
−	....	1800	1850	1920
−	....	1955	2270	2135
	B. Mn^{54} added at 3rd wk of the expt			
+	1900	1700	1770	1900
+	1800	1460	1490	....
−	320 **	460 **	1120	1260
−	230 **	330 **	1200	1500

* Cotyledons were used for radioautograph studies.
** Counts were so close to background that they have no significance.
Hoagland solution, 20%; Mn conc, 5 ppm. Data reported as cpm/gm.

Radioactivity of barley leaves in counts per minute per gram of oven dried leaf tissue is reported in table III. The counts were made on the cotyledons and the 1st, 2nd, and 3rd leaves of the plants grown in the previous experiments. The cotyledons and the 4th, 5th, and 6th leaves of plants grown in Mn^{54} from the beginning of the experiment were not counted for Mn^{54} as they had been used in the prep-

aration of radioautographs. The dried leaves were cut into sections and placed in a small dish under the counting tube of the Geiger counter. While this does not give a precise count of all of the radioactivity, it allows a comparative evaluation between treatments. The data show a slightly higher concentration of Mn in the 1st leaf of the Si treated plants than in the 2nd and 3rd leaves. This is in line with previous data (table I) which showed older leaves accumulating more Mn than younger leaves. The 1st leaf of plants grown in the presence of silicon contained more Mn than a similar leaf from plants grown in the absence of silicon. This may have been due to an early development of necrosis on the leaves from plants grown in the non-silicon treatments producing a rapid desiccation and early death of the leaf as compared to the silicon treated plants whose leaves were still green and healthy at the time of harvest. There was no noticeable difference in the Mn content of the 2nd and 3rd leaves between treatments or within a treatment. These data indicate that plants were able to continue supplying Mn even to old leaves as long as the tissues were healthy.

In section B of the table the counts are given on leaves from plants to which Mn^{54} was added 3 weeks after the seedlings were placed in the culture solutions. In all treatments 5 leaves were present on the original stock and at least 1 or 2 suckers had developed at the time the Mn^{54} was added to the solutions. The leaves on the Si treated plants were all green and healthy at the time of radioactivity addition whereas in the non-silicon plants the cotyledons and the 1st leaf were heavily spotted, the 2nd leaf was moderately to heavily spotted, and the 3rd leaf had spots 1 to 2 inches down the leaf from the tip. As may be observed in the table, Mn moved into the cotyledon and the 1st and 2nd leaves of the silicon treated plants in much greater quantity than went into the leaves from the non-silicon treated plants which had been heavily spotted at the time the Mn^{54} was added to the solutions. The less heavily spotted leaves took up more Mn than did those which had been heavily spotted. At the time of harvest the cotyledons and the first 4 leaves of the original stock of the plants grown in the absence of Si were dried up and dead whereas all of the leaves from the plants grown in the presence of silicon were green and healthy. The data for the counts on the cotyledon and 1st leaf of the non-silicon treatment are questionable in as much as the count was only slightly greater than observed for background.

Discussion and Summary

Mn was found to be toxic to barley plants when grown in Hoagland culture solutions at concentrations from 0.5 to 5.0 ppm. The necrotic symptoms which develop are small dark brown spots which first appear at the tips of the older leaves and spread down the leaf until the entire blade is covered. This necrosis may be alleviated by increasing the macro-salt concentration, decreasing the micro-salt concentration, or by the addition of soluble silicates to the culture solutions. In all cases a decrease in the amount of necrosis is associated with an increase in yield. Unlike Ca, Mg, and K which eliminate Mn toxicity by repressing the amount of Mn moving into the leaf, silicon has no effect on the content of Mn in the leaf tissue.

Radioautographs of the leaves of soybean, tomato, and alfalfa removed from plants grown in Mn^{54} were made by Romney and Toth (4). Small islands of radioactivity were observed in the older leaves with decreasing amounts in younger leaves. Similar autographs were made by the authors on the leaves of barley plants grown in culture solutions containing Mn^{54} in the presence and absence of Si. A comparison of the radioautographs with photographs of the same leaf areas showed a direct correlation between the presence of radioactive islands and the visible necrotic spots. Radioautographs of leaves from plants grown in the presence of Si showed no islands of concentrated radioactivity as were observed in its absence. The radioactivity in barley leaves from plants grown in the presence of silicon was shown to be present in a uniform distribution throughout the leaf.

When Mn^{54} was added to the plants after they had been growing in culture solutions for 3 weeks it was noted that the radioactivity moved freely into the green healthy leaves but not into leaves which were necrotic and nearly dead at the time of addition of the radioactive Mn.

The beneficial effect of silicon on the yield of barley plants grown in standard Hoagland solutions is due to a secondary effect and is not a result of the overcoming of a deficiency of silicon. Silicon alters the distribution of Mn in the leaf tissue rather than excluding it from the plant as occurs in the presence of high concentrations of macro-salt. The unique effect of Si in preventing the accumulation of Mn in localized toxic concentrations is of great value in preventing necrosis due to Mn toxicity. However, the exact mechanism by which Si accomplishes this phenomenon is as yet unknown. These experiments, of course, do not preclude the essentiality of silicon for higher plants. They demonstrate alternative ways of achieving the beneficial effect obtained by the addition of silicon in the reduction of manganese toxicity in barley leaves.

The authors are indebted to John B. Capindale of the Department of Soils and Plant Nutrition for the preparation of the radioautographs used in this paper.

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KREBS CYCLE ACTIVITY OF MITOCHONDRIA FROM ENDOSPERM OF SUGAR PINE SEED (PINUS LAMBERTIANA DOUGL.)[1]

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Previous studies on the enzyme activities associated with plant particulate fractions have been limited to germinating seedlings or to storage structures derived from Angiosperm type plants (5, 10). The preceding publication described some properties of mitochondria from seedlings and embryos of *Pinus lambertiana*, a Gymnosperm (15). In this paper these studies have been extended to pine endosperm, a type of tissue not yet investigated.

Although the endosperm of Gymnosperm seeds is functionally analogous to the endosperm of Angiosperm seeds, it is of different origin and genetic constitution (3). Therefore, only the desire to maintain a simplified nomenclature justifies the use of the term endosperm. Pine endosperm is haploid female gametophyte tissue, and does not result from a fusion between two or more nuclei as is the case in Angiosperms. This difference should be borne in mind in attempting to compare the results reported here to those from other plant tissues.

Metabolic and radioactive tracer studies were employed to obtain evidence for the Krebs cycle enzyme system in mitochondria isolated from homogenates of pine endosperm. Ungerminated and germinating sugar pine seeds were used for these studies. Some variations in the oxidative enzyme activity associated with these particles during the first ten days of germination are presented.

Materials and Methods

The majority of the studies were carried out on seeds harvested in September, 1954 and 1956, at Pino Grande, near Placerville, California. However, the seeds used in the initial experiments establishing the presence of the Krebs cycle were harvested in 1953 in the same vicinity. The cones were dried in a circulating air drying oven at 70° C until the cone scales had reflexed and the seeds had dropped out or could be shaken out. Seeds were then dewinged, cleaned by hand, and placed in a sealed glass jar which contained $CaCl_2$ as a drying agent. The seeds were stored at 5° C until used.

In most of the experiments reported here, mitochondria were isolated from the endosperm of ungerminated seeds. The seeds had been soaked for six hours in aerated water at 5° C before the embryo and endosperm were separated. In a few experiments (fig 1) the endosperm was obtained from germinating seeds at daily intervals. In those experiments the

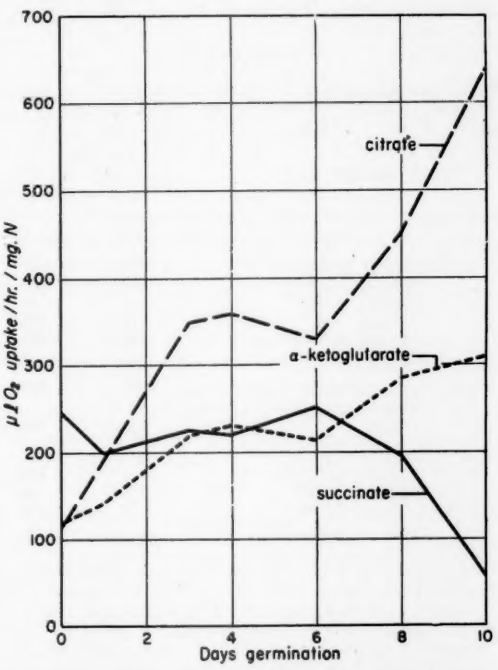

Fig. 1. Changes in the rate of substrate oxidation by endosperm mitochondria. Conditions were those employed in table I.

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[4] Maintained at Berkeley, California by the Forest Service, U. S. Department of Agriculture, in cooperation with the University of California.

seeds were prepared and germinated as previously described (15).

Samples of endosperm weighing 50 to 75 grams were washed in a weak solution of Duponol c to remove surface contaminants, and rinsed five times in sterile distilled water. This amount of endosperm was readily obtained, free of embryo, from about 300 seeds. The mitochondria were then isolated using the procedure employed in the studies on the embryo of the same species (15). The reagents and methods employed in measuring oxidative respiration, radioactive carbon dioxide, and the nitrogen and organic acid assays were also the same as used in those studies.

Results

KREBS CYCLE ENZYME ACTIVITY OF THE PARTICULATE FRACTION: The cofactors required for the oxidation of succinate and citrate by mitochondria from the endosperm of sugar pine seed are presented in table I. In general, the oxidation of citrate was affected more by the omission of various cofactors than was the oxidation of succinate. In particular, the oxidation of citrate was decreased by the omission of $MgSO_4$ and cytochrome c, whereas succinate oxidation was unaffected. The omission of AMP decreased the oxidation of both substrates, and ATP was able to substitute for AMP.

The omission of a commercial coenzyme concentrate, containing DPN^+, TPN^+, and CoA, also lowered the oxidation of citrate. An analysis of this effect demonstrated that DPN^+ was the chief component of the coenzyme mixture which was essential for maximum oxidation of citrate.

TABLE I

EFFECT OF COFACTORS ON OXIDATIVE ACTIVITY OF MITOCHONDRIA FROM ENDOSPERM OF PINUS LAMBERTIANA

Expt. no.	Conditions	Substrate	
		Succinate	Citrate
		$\mu l\ O_2/hr \times mg\ N$	
1	Complete system	545	146
	without $MgSO_4$	550	100
	without AMP	404	60
	without AMP; add ATP	547	145
	without cytochrome c	548	116
	without substrate	4	4
2	with coenzyme concentrate	405	232
	without coenzyme concentrate	405	121
3	Complete system	...	187
	without coenzyme A	...	180
	without DPN^+	...	86
	without TPN^+	...	176
	without CoA, DPN^+, TPN^+	...	74

Complete reaction mixture contained 10 micromoles $MgSO_4$, 1 micromole AMP or ATP, 0.1 micromole cytochrome c and 30 micromoles of succinate or citrate in 0.4 M sucrose + 0.02 M phosphate buffer, pH 7.0. Total volume, 2.5 ml; temperature 30° C. The center well contained 0.1 ml 5 M KOH and filter paper. Substrates were adjusted to pH 7.0.

In experiment 3 the complete mixture also contained 1.2 micromoles Coenzyme A; 1.8 micromoles DPN^+; and 0.9 micromole TPN^+.

TABLE II

OXIDATION OF PYRUVATE-2-C^{14} AND ACETATE-2-C^{14} BY MITOCHONDRIA FROM ENDOSPERM OF PINUS LAMBERTIANA

Substrate added	Initial activity	Activity recovered in CO_2
	cpm	%
Pyruvate-2-C^{14}	91,000	13.1
Pyruvate-2-C^{14} *	91,000	0.0
Acetate-2-C^{14}	90,000	9.3
Acetate-2-C^{14} *	90,000	0.0

Each flask contained mitochondria from endosperm of seedlings germinated 8 days, 21 micromoles pyruvate or 0.4 micromole acetate; 2.0 micromoles malate; 0.6 micromole Coenzyme A; 0.9 micromole DPN^+; 0.5 micromole TPN^+; 0.8 micromole TPP; 0.05 micromole cytochrome c; 5 micromoles $MgSO_4$; and 5.2 micromoles AMP in 0.4 M sucrose + 0.02 M phosphate buffer, pH 7.0. The flasks with acetate-2-C^{14} also contained 0.04 mg lipoic acid. Total volume, 1.2 ml. The center well contained 0.04 ml 5.0 M KOH; the side arm, 0.15 ml 10 *N* H_2SO_4. Substrates were adjusted to pH 7.0. Incubated 1.6 hrs at 25° C.

* Mitochondria were heat inactivated.

In the presence of a complete cofactor system the mitochondria from endosperm of ungerminated seeds were also able to oxidize other intermediates of the Krebs cycle. The substrates oxidized and the rates of oxidation observed, $Q_{O_2}(N)$, in a typical experiment were: succinate, 320; citrate, 120; α-ketoglutarate, 130; cis-aconitate, 45; L-malate, 60; fumarate, 59; and pyruvate (in the presence of catalytic amounts of L-malate), 105. Inhibition of the oxidation of these substrates in the presence of 20 micromoles of malonate ranged from 35 % for citrate to 88 % for succinate.

When pyruvate-2-C^{14} was added to a preparation of mitochondrial particles from the endosperm of sugar pine seeds, the labeled pyruvate was metabolized and $C^{14}O_2$ was released (table II). The release of $C^{14}O_2$ from pyruvate-2-C^{14} suggests that pyruvate, after initial decarboxylation, is degraded by way of the Krebs cycle. To confirm that this was the case, intermediates of the Krebs cycle were isolated from the reaction mixture by chromatography and observed for radioactivity. Radioautographs of the chromatograms showed that citric, malic, and succinic acids were radioactive.

To gain more direct evidence for the initial step of the Krebs cycle, the oxidation of acetate-2-C^{14} was investigated. Table II shows that the addition of $C^{14}H_3COOH$ to mitochondria from the endosperm gave rise to radioactive CO_2. Such reactions have been observed in animal mitochondria (7).

Two additional properties of pine endosperm mitochondria, which permit further comparison of the oxidative responses of these particles to mitochondria from other plants, were observed. As with mito-

chondria from pine seedlings (15), particles from endosperm exhibited highest oxidation rates when 0.4 M sucrose and 0.02 M phosphate buffer, pH 7.0 was employed in the reaction mixture. Phosphate (0.1 M, pH 7.0) was also required in the grinding media in order to obtain maximum oxidation rates by these mitochondria. This finding is similar to the results of Millerd et al (11) with mung bean particles and the results of Beaudreau and Remmert (1) with bean mitochondria.

Changes in Krebs Cycle Enzyme Activity of Mitochondria From Endosperm During Germination: Although the capacity to oxidize intermediates of the Krebs cycle is inherently present in the ungerminated seed, the initial activity was related to the amount of water which the seed had absorbed. Results in table III demonstrate that mitochondria from the endosperm of seeds soaked for six hours in water were ten times as active, on a nitrogen basis, as mitochondria from endosperm of seed which had not been soaked in water.

Since the endosperm is greatly reduced in weight and is discarded by the developing seedling shortly after growth begins, it was of interest to follow the changes in the different enzyme activities of the endosperm during germination. Figure 1 indicates that significant changes in enzyme activity occurred during the first ten days of germination. During this period the capacity of endosperm mitochondria to oxidize citrate increased five fold while the capacity to oxidize α-ketoglutarate increased approximately two fold. On the other hand, the oxidation of succinate by the mitochondria remained constant for 6 days and then markedly decreased. The respiration in the absence of added substrate was 0 to 5 μl per hour. Lieberman and Biale have shown that an apparent decrease in the mitochondrial succinoxidase activity of broccoli buds can be restored by grinding in a medium containing ethylenediaminotetraacetic acid and sucrose (9). This treatment did not effect an increase in succinate oxidation by mitochondria from sugar pine endosperm.

Table III

Effect of Soaking Seeds on Oxidative Activity of Endosperm Mitochondria

Time of soaking	Water content of endosperm	Substrate oxidized		
		Succinate	α-Keto-glutarate	Citrate
hrs	%	$\mu l\ O_2/hr \times mg\ N$		
0	3	37	11	0
2	32	86	33	32
6	57	246	118	112

Conditions for measuring O_2 uptake are described in table I. Substrate concentration, 30 micromoles per flask. The soaking of the seeds was carried out as described under Methods. The endosperm water content represents the mean of ten determinations obtained by drying to a constant weight at 90° C.

Discussion

From the limited number of studies of endosperm respiration thus far reported (2, 13), one might conclude that the nutritive tissue present in germinating Angiosperm seeds contains a mechanism for aerobic respiration similar to that found in the maturing sporophyte. The present study extends this concept to include the nutritive tissue sustaining the initial seedling development of a member of the Gymnosperms. The haploid endosperm (female gametophyte) of sugar pine seed was shown to contain the same oxidative pathway as was found in the diploid pine embryo and seedling (15), and in the triploid endosperm of castor bean (2). This study also provides a basis for consideration of the fundamental question: Is endosperm respiration due to the enzyme activity of the endosperm protoplasm, or to the secretion of enzymes into it from the embryo? This is a question that has frequently been asked (3, 12). In sugar pine seed, it appears that all of the necessary respiratory enzymes are present initially in the endosperm tissue.

The relationship between the water content and enzyme activity of the endosperm reported here (table III) may provide certain basic physiological insights concerning seed germination. Laties has demonstrated the inhibitory effect of high osmotic concentrations on particulate enzyme activity (8). Similar osmotic effects were noted with mitochondria from the endosperm of sugar pine. During the germination period the endosperm tissue of pine absorbs water to a given level, and then remains at this level throughout the greater portion of its functioning period (14). We may speculate that this level of water provides the proper intracellular environment essential to the functioning of the particulate enzyme units. Jansson (6) also observed a high degree of correlation between dehydrogenase activity and moisture content in germinating barley seeds. These results appear to substantiate the opinion of Toole et al (17) that "One of the early phases of the seed germination process must be the activation of the mitochondria enzyme systems."

The capacity of mitochondria to metabolize increased amounts of citrate over succinate (fig 1) may be the result of changes in the absolute amounts of these enzymes, in their solubilities, or in their turnover rates. Other workers have reported a decrease in particulate succinoxidase activity in developing barley and bean seedlings (4, 16). A concomitant increase in endogenous respiration was also noted in these latter studies. This endogenous increase was not detected with mitochrondria from sugar pine endosperm.

Summary

1. Mitochondria from the haploid endosperm (female gametophyte) of sugar pine contain the enzymes of the Krebs cycle. Of the cofactors tested, only AMP was required for maximum oxidation of

succinate, while Mg^{++}, AMP, and DPN^{+} were required for the oxidation of citrate.

2. Pyruvate-2-C^{14} and acetate-2-C^{14} were metabolized by the mitochondria.

3. The absorption of water by the endosperm tissue was accompanied by the activation of the Krebs cycle enzyme system.

4. The capacity of mitochondrial particles to oxidize succinate decreased as the embryos germinated; their ability to oxidize citrate and α-ketoglutarate increased.

5. The possible physiological significance of changes in particulate enzyme activity, as related to water uptake by the germinating seed, is discussed.

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ENZYME ACTIVITY OF MITOCHONDRIA FROM GERMINATING SEEDLINGS OF SUGAR PINE (PINUS LAMBERTIANA DOUGL.)[1]

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Recent studies of the respiratory activity of plant mitochondria leave little doubt of the importance of these cellular units in plant metabolism. Several papers have described the oxidative activities of mitochondria prepared from Angiosperm species and the lower groups, bacteria, fungi and algae (1, 15, 29). Two excellent reviews have recently appeared (8, 10). However, no work on the oxidative nature of particles from a Gymnosperm species has yet been reported. This paper will describe experiments concerned with the oxidative activities of particulate fractions obtained from germinated seedlings and ungerminated embryos of *Pinus lambertiana*, a Gymnosperm.

Changes in the levels of enzyme activities and metabolic routes in maturing plants have been reported by several workers. Gibbs and Beevers (7) detected differences in the pathway of glucose dissimilation in tissues of varying ages from castor bean, pea, and other plants. Brummond and Burris (5) noted changes in the Krebs cycle enzymes associated

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with mitochondrial particles from green leaves of lupine. Beevers and Walker (4), using mitochondria from the endosperm of germinated castor bean, showed significant changes to occur in the levels of succinate oxidation associated with particles removed from tissues of different ages. This paper will present evidence for a decrease in the activities of several Krebs cycle enzymes during the germination of sugar pine seed. A soluble cytoplasmic factor possibly effecting such changes will be discussed.

Materials and Methods

The majority of these studies used sugar pine seed harvested in September, 1954, at Pino-Grande near Placerville, California. This seed was supplied by the Institute of Forest Genetics, U. S. Forest Service, Placerville. The initial experiments, which established the presence of the Krebs cycle, used seeds harvestd in 1953 in the same general vicinity. The cones were dried in a circulating air drying oven at 70° C until the cone scales had reflexed and the seeds dropped out or could be shaken out. Seeds were then dewinged, cleaned by hand, and placed in a sealed glass jar which contained $CaCl_2$ as a drying agent. The seeds were stored at 5° C until used.

Seeds were surface sterilized by soaking in a 0.75 % bromine-water solution for five minutes. They were rinsed five times in sterile distilled water and allowed to stand in sterile water for ten minutes before the hard seed coat, papery membrane and nucellar cap were aseptically removed. The divested seeds, containing the embryo surrounded by the female gametophyte tissue were then placed in sterile distilled water at 5° C and air was continuously bubbled through the water for six hours. At the end of six hours the seeds were planted 1/4 to 1/2 inch below the surface of flats of sterile vermiculite. Enough water was added daily to maintain a constant amount of moisture available to the germinating seedlings. After five or six days at 30° C ± 1° C the radicle protruded about 2 cm and the hypocotyl had grown about 0.5 cm. This germinated seedling, separated from residual female gametophyte tissue, was the material used in most of the experiments. When ungerminated embryos were employed (tables IV, VI and VII) the embryos were removed from the seed after six hours of soaking. The separation of the embryo from the surrounding female gametophyte tissue was complete.

Embryos from stratified ungerminated seeds were used in some experiments (table VI). To obtain these embryos, intact seeds were stratified without preliminary soaking. They were mixed with moist vermiculite and packed in waxed paper quart milk containers which were stored at 5° C for varying intervals of time up to four months. The embryos were then separated from the surrounding female gametophyte tissue without preliminary soaking of the seed.

Isolation of Particles and Measurement of Activity: Forty to 50 gm samples of germinated seedlings were rinsed five times in sterile distilled water. They were then transferred to a chilled mortar and homogenized with sand in 150 ml 0.5 M sucrose and 0.1 M phosphate buffer, pH 7.0, for approximately 30 seconds. The pH was maintained at 7.0 with 1.0 M NaOH during grinding. The homogenate was filtered through two layers of cheese cloth, and the filtrate was centrifuged for five minutes at 2,000 × g in an International refrigerated centrifuge. The supernatant solution was then centrifuged at 20,000 × g for 15 minutes. The precipitate obtained was washed by resuspending in 10 ml of 0.5 M sucrose and 0.1 M phosphate buffer, pH 7.0, and centrifuging at 20,000 × g for 15 minutes. The precipitate was suspended in sucrose-phosphate buffer of such concentration that, when 0.5 ml aliquots of the particulate suspension were added to the reaction vessels, the final concentration of sucrose and phosphate in the flasks was 0.4 and 0.02 M, respectively. The final suspension contained 1.0 to 4.0 mg of nitrogen per ml. All preparative steps were carried out at 0 to 5° C.

The particulate fraction containing the microsomes was prepared by centrifuging the supernatant solution, from which the mitochondria had been removed, at 100,000 × g for one hour. The microsomal pellet obtained was then suspended in 0.5 M sucrose and 0.1 M phosphate, pH 7.0.

When ungerminated embryos were used as a source of mitochondria, about 15 gm of embryos were homogenized in 50 ml of 0.5 M sucrose and 0.1 M phosphate, pH 7.0.

Unless otherwise stated, manometric measurements were carried out in air in Warburg flasks of approximately 6 ml volume; the center well contained 0.04 ml of 5 M KOH and filter paper. After mitochondria were added to complete the reaction mixture, the flasks were attached to manometers, immersed in a water bath at 25° C, and allowed to equilibrate for ten minutes. Oxygen consumption in the presence of various substrates was then compared to that in the absence of substrates. Values of oxygen uptake, expressed as microliters per hour or microliters per hour per mg of mitochondrial nitrogen $Q_{O_2}(N)$, were calculated on the basis of the reaction occurring during the first twenty minutes.

The nitrogen content of the mitochondrial preparations was determined by digestion with 12 *N* H_2SO_4 containing $CuSO_4$ and Na_2SeO_3. The ammonia was measured by direct Nesslerization.

The amount of $C^{14}O_2$ produced metabolically by particles respiring radioactive substrates was determined by the method of Humphreys, et al (12).

The separation and identification of Krebs cycle acids was done by paper chromatographic procedures. After removal of the KOH, the reaction mixtures were transferred to centrifuge tubes. Two volumes of absolute ethanol were added to precipitate the protein fraction. The precipitate was then boiled in 10 ml of absolute ethanol to remove any adsorbed organic acid, and this ethanol solution was added to the supernatant solution from the protein precipitation. The

ethanol solution was then evaporated to dryness, the residue redissolved, and the organic acids extracted by a $KHSO_4$ column (24). The ether eluent was concentrated to 0.5 ml and placed on 6×24-inch strips of Whatman No. 1 filter paper either as a spot or band. Spots of known acids were placed adjacent to the unknown. Descending chromatograms were developed with *n*-butanol–formic acid–water (16). The chromatograms were dried and placed in contact with Kodak no-screen x-ray film for 2 to 5 days, and the radioautographs developed. The chromatograms were eluted with water, the eluent concentrated and rechromatographed with the suspected intermediates at adjacent spots. Radioautographs of the rechromatographed acids were prepared, and the labeled acids identified from the adjacent known acids.

REAGENTS: Sodium pyruvate and α-ketoglutaric acid were obtained from Nutritional Biochemical Corporation. Fumaric acid (recrystallized) was obtained from Dr. L. Ordin. Citric, L-malic, and succinic acids were obtained from Eastman Kodak Co.

The following cofactors were purchased from Nutritional Biochemical Corporation: diphosphopyridine nucleotide (DPN^+), 90 %; triphosphopyridine nucleotide (TPN^+), 95 %; liver coenzyme concentrate containing 4 % DPN^+, 7 % TPN^+, and 10 Lipmann units Coenzyme A per mg; adenosine-5-phosphoric acid (AMP); adenosine triphosphate (ATP); Coenzyme A (CoA), 70 %; and thiamine pyrophosphate (TPP). Cytochrome c was obtained from Sigma Chemical Corp.; lipoic acid (Lederle) was obtained from Dr. P. K. Stumpf.

Pyruvamide-2-C^{14} and acetate-2-C^{14} were obtained from Tracerlab, Inc. Non-labeled pyruvamide was the gift of Dr. B. Vennesland. Alanine-1-C^{14}, alanine-3-C^{14}, palmitate-1-C^{14} and butyrate-1-C^{14} were obtained from Dr. P. K. Stumpf. Sucrose of commercial grade was used. Deionized distilled water was used in all metabolic experiments.

RESULTS

OXIDATION OF KREBS CYCLE INTERMEDIATES: In initiating these studies, suitable conditions had to be selected for isolating mitochondria from seedlings of *Pinus lambertiana* and measuring their enzymatic activities. The procedures developed by other workers with plant mitochondria served as a guide (10). In preliminary studies in which the oxidation of α-ketoglutarate and succinate were investigated, homogenization of the seedling with sand in a mortar with pestle proved superior to a chilled Waring blender run at maximum speed for 10 seconds. Highest rates of oxidation were obtained when the seedlings were homogenized in 0.5 M sucrose and 0.1 M phosphate, pH 7.0, and when the activity of the particles was measured in reaction mixtures containing 0.4 M sucrose and 0.02 M phosphate, pH 7.0. It was necessary to maintain the pH at 7.0 both during homogenization and later during measurement of enzymatic activity. As was anticipated, magnesium ions and either AMP or ATP were required to obtain maximum rates of oxidation of α-ketoglutarate and succinate by the pine particles. Cytochrome c had no effect on the rate of oxidation of these substrates. However, cytochrome c and four cofactors (DPN^+, TPN^+, TPP and CoA) were frequently added to reaction mixtures to prevent the reaction rates being limited by insufficient concentrations of these substances.

TABLE I

OXIDATION OF KREBS CYCLE SUBSTRATES BY MITOCHONDRIA FROM PINUS LAMBERTIANA SEEDLINGS

SUBSTRATE	MICROMOLES/VESSEL	$Q_{O_2}(N)$
None	..	8
Succinate	20	346
Citrate	30	156
α-Ketoglutarate	30	111
Fumarate	30	100
L-Malate	30	100
L-Malate	2	27
Pyruvate	17	16
L-Malate + Pyruvate	2 + 17	139

In addition to the substrates the flasks also contained 0.5 ml mitochondria suspension; 5 micromoles $MgSO_4$; 7.0 micromoles AMP; 0.05 micromoles cytochrome c in 0.4 M sucrose + 0.02 M phosphate buffer, pH 7.0. The flasks with malate and pyruvate contained, in addition, 0.6 micromoles CoA; 0.9 micromoles DPN^+; 0.5 micromoles TPN^+; 0.8 micromoles TPP; total volume of fluid was 1.2 ml. All substrates were adjusted to pH 7.0.

The ability of mitochondria isolated from 5- or 6-day-old seedlings of *Pinus lambertiana* to catalyze the oxidation of various Krebs cycle substrates is shown in table I. All the substrates tested were oxidized. The $Q_{O_2}(N)$ for the various substrates were of the same order of magnitude as reported for other plant mitochondria (4). The characteristic catalytic action of malate on pyruvate oxidation described by Millerd et al (17, 18) was also noted. Particles sedimenting between 20,000 and $100{,}000 \times g$ did not catalyze the oxidation of Krebs cycle intermediates.

To determine if oxidation of the acids was pro-

TABLE II

MALONATE INHIBITION OF OXIDATIONS CATALYZED BY MITOCHONDRIA FROM PINUS LAMBERTIANA SEEDLINGS

SUBSTRATE ADDED	CONCENTRATION	OXYGEN UPTAKE		INHIBITION
		− MALONATE	+ MALONATE (20 MICROMOLES)	
	micromoles/vessel	*μl/20 min*		*%*
None	..	2	4	..
Succinate	20	81	21	74
L-Malate	2	15	8	47
L-Malate and Pyruvate	2 14	53	29	45
α-Ketoglutarate	20	51	17	67

Conditions are as described for table I.

ceeding through a common step, malonate was added to reaction mixtures which had respired twenty minutes on various Krebs cycle substrates. The readings were resumed five minutes after tipping in the inhibitor from the side arm. Under these conditions, malonate inhibited the oxidation of pyruvate, succinate, α-ketoglutarate, and L-malate by the mitochondria (table II). Since malonate is known to be an effective inhibitor of succinic dehydrogenase (14), we may conclude that the oxidation of malate, pyruvate, and α-ketoglutarate proceeded through the common intermediate, succinate.

METABOLISM OF PYRUVATE-2-C^{14}, ACETATE-2-C^{14}, ALANINE-1-C^{14}, AND ALANINE-3-C^{14}: When pyruvate-2-C^{14} or acetate-2-C^{14} was added to mitochondrial preparations, the particles metabolized the labeled substrates and the CO_2 released was radioactive. As shown in table III, the amount of radioactive carbon recovered as CO_2 ranged from 5 to 20 % of the amount added initially. Radioautograms showed that malic, citric, and succinic acids isolated from the reaction mixtures were radioactive.

TABLE III

METABOLISM OF PYRUVATE-2-C^{14} AND ACETATE-2-C^{14} BY MITOCHONDRIA FROM PINUS LAMBERTIANA SEEDLINGS

EXPT. NO.	MITOCHONDRIAL N ADDED	INITIAL ACTIVITY IN PYRUVATE	INITIAL ACTIVITY IN ACETATE	ACTIVITY RECOVERED AS CO_2
	mg	*cpm × 10³*	*cpm × 10³*	*%*
158	1.3	54	...	13.8
154	0.9	90	...	6.8
154	0.9 (heated)	90	...	0.0
158	1.3	..	144	20.4
154	0.9	..	90	5.5
154	0.9 (heated)	..	90	0.0

Each flask contained 14 micromoles pyruvate and 2 micromoles of malate, or 0.4 micromoles acetate and 2 micromoles of malate added initially. All other conditions and cofactors were as listed in table I, except that filter paper was omitted from the center well. The side arm contained 0.1 ml 10 *N* H_2SO_4 which was tipped into the main compartment at the conclusion of the experiment. The flasks containing acetate-2-C^{14} also contained 0.04 mg lipoic acid. Incubated 2 hrs at 25° C.

Recent work has indicated that plant mitochondria possess transaminase activity (2, 26, 28). By adding alanine-1-C^{14} or alanine-3-C^{14} and α-ketoglutarate to mitochondria from germinated sugar pine seedlings or ungerminated embryos, it was possible to demonstrate the evolution of $C^{14}O_2$ (table IV). In the case of mitochondria from ungerminated embryos, the production of $C^{14}O_2$ was only partially dependent upon the presence of a keto acid. It is possible, therefore, that alanine oxidation occurred by processes involving reactions other than transamination to pyruvate. However, citric, pyruvic, and succinic acids isolated from the reaction mixture were found to be radioactive.

TABLE IV

METABOLISM OF ALANINE-1-C^{14} AND ALANINE-3-C^{14} BY MITOCHONDRIA FROM PINUS LAMBERTIANA

SOURCE OF MITOCHONDRIA	SUBSTRATE AND INITIAL ACTIVITY	CPM IN CO_2	% ACTIVITY RECOVERED AS CO_2
Seedling	Alanine-3-C^{14} (54,000 cpm)	5,250	9.7
Seedling (heated)	Alanine-3-C^{14} (54,000 cpm)	28	0.05
Ungerminated embryo	Alanine-1-C^{14} (38,200 cpm)	19,420	51.0
Ungerminated embryo	Alanine-1-C^{14} * (38,200 cpm)	14,700	38.5
Ungerminated embryo (heated)	Alanine-1-C^{14} (38,200 cpm)	900	2.4

Conditions as in table III except that substrate was radioactive alanine (2.6 micromoles) instead of pyruvate or acetate. In addition, each vessel contained 30 micromoles of α-ketoglutarate instead of malate. Incubated 2.5 hours at 25° C.

* Omit α-ketoglutarate.

METABOLISM OF FATTY ACIDS: Studies of the pathways of fatty acid oxidation have shown the essential enzymes to be associated either with mitochondria in animal tissues (9), or with mitochondrial and microsomal particles in the peanut (13, 25). It was, therefore, of interest to determine where such activity exists in the pine seedling. Data presented in table V show that both mitochondrial and microsomal particles catalyzed the formation of $C^{14}O_2$ from palmitate-1-C^{14}. Butyrate-1-C^{14} was also metabolized by mitochondria from seedlings of *P. lambertiana*. In view of the work of Newcomb and Stumpf (21) on long chain fatty acid oxidation by peanut

TABLE V

METABOLISM OF PALMITATE-1-C^{14} AND BUTYRATE-1-C^{14} BY PARTICULATE FRACTIONS FROM PINUS LAMBERTIANA SEEDLINGS

PARTICLES	INITIAL ACTIVITY IN PALMITATE-1-C^{14}	INITIAL ACTIVITY IN BUTYRATE-1-C^{14}	ACTIVITY RECOVERED AS CO_2
	cpm	*cpm*	*%*
Mitochondria	1,900	....	7.0
Mitochondria (heated)	1,900	....	0.0
Microsomes	1,900	....	7.8
Microsomes (heated)	1,900	....	0.0
Mitochondria	....	7,500	8.5
Mitochondria (heated)	....	7,500	0.0

Each flask receiving palmitate (0.1 micromole) also contained 0.5 ml mitochondrial suspension; 0.4 M sucrose + 0.02 M phosphate buffer, pH 7.0; 10 micromoles $MgSO_4$; 11 micromoles AMP; 0.1 micromole cytochrome c. The side arm contained 0.3 ml 10 *N* H_2SO_4; the center well contained 0.2 ml 5 M KOH; total volume of reaction mixture 3.0 ml; volume of vessels, about 15 ml. Incubation time, 1 hr. The flasks receiving butyrate-1-C^{14} (0.1 micromole) in addition contained 0.8 micromole TPP; 0.6 micromole CoA; 0.9 micromole DPN^+; 0.5 micromole TPN^+; and 15 micromoles malonate.

microsomes, it appeared that the pine seedling mitochondria might be contaminated with smaller particles. Contamination was possible because some agglutination and sedimentation of the microsomes might have occurred in the sucrose-phosphate medium used for extraction (13). However, repeated washing of the mitochondrial preparations of pine seedlings did not diminish their ability to metabolize palmitate-1-C^{14}. The recent investigation by Stumpf and Barber (25) of the capacity of peanut mitochondria to metabolize fatty acids shows that mitochondria of peanut seedling cotyledons also contain enzyme systems for the β-oxidation of fatty acids.

Changes in Krebs Cycle Enzyme Activity During Stratification and Germination: As time of stratification increases up to three or four months, seeds of many plants increase in capacity and rate of germination (6). Mirov has shown this to be a characteristic of sugar pine seed (19). Therefore it was desirable to compare the enzymatic activity of mitochondria isolated from embryos of seeds subjected to different periods of stratification. Also, the activity of these particles was compared to that of particles extracted from the non-stratified, germinated sugar pine seedlings used in most of the present studies. These results are summarized in table VI.

The mitochondria from embryos of non-stratified, ungerminated seeds possessed a higher level of Krebs cycle enzyme activity, expressed as $Q_{O_2}(N)$, than the mitochondria from germinated seedlings. The values in table VI were obtained with seeds harvested in 1954. It can be seen that, upon germination, the rate of oxidation of Krebs cycle substrates decreased about 60 %. These results are typical of those obtained in a dozen or more experiments with 1954 seed. In addition, mitochondria from seeds harvested in 1956 showed a decrease in rate of oxidation of about 80 % after 9 days of germination. Table VI also shows that the mitochondria from embryos of non-stratified, ungerminated seeds possessed a higher level of enzyme activity, per mg N, than mitochondria from embryos of seeds stratified for 120 days. The results obtained with mitochondria from seeds stratified for only 60 days were intermediate in value.

Some preliminary experiments were performed in an attempt to explain the decreased activities of the mitochondria from germinated seedlings. The supernatant solution from germinated seedlings from which the mitochondria were removed did not decrease the activity of mitochondria from ungerminated embryos. This would appear to exclude the presence of an inhibitor in this supernatant. The supernatant solution from the germinated seedlings had no effect on the mitochrondria from the germinated seedling. On the other hand, the supernatant from ungerminated embryos had a large stimulatory effect on the mitochondrial particles from germinated seedlings (table VII). The stimulatory effect was not lost when the supernatant was boiled for five minutes at neutrality, but it was lost when the supernatant was boiled for five minutes in either 1 M HCl or 1 M KOH. Neither the microsomes nor the ashed residue of the supernatant liquid stimulated oxidation. The possibility of the stimulatory component being an oxidizable substrate or one of the cofactors of the Krebs cycle was examined. Doubling the concentration of succinate, citrate, and all of the added cofactors did not increase the rate of oxidation of succinate or citrate in the absence of the supernatant. The addition of flavin coenzymes (flavin mononucleotide and

Table VI

Effect of Germination and Stratification on Oxidative Activity of Mitochondria from Pinus lambertiana Embryos and Seedlings

Substrate	Conc, micromoles/vessel	Seed treatment: Not stratified, Ungerm'd	Not stratified, Germ'd, 5 days *	Stratified, ungerm'd, 60 Days	Stratified, ungerm'd, 120 Days
		Oxygen uptake, $\mu l/hr \times mg\ N$			
Succinate	20	880	330	720	310
Citrate	30	410	180	300	220
α-Ketoglutarate	30	470	200	210	210
L-Malate	20	330	180	320	150
L-Malate	2	80	55	100	50
Pyruvate	17	140	10	120	40
Pyruvate + L-malate	17 + 2	420	220	310	160

Conditions were as described for table I.
* This treatment corresponds to the standard germination procedure described under Methods.

Table VII

Effect of Supernatant Fractions on Oxidative Activity of Mitochondria from Germinated Seedlings

Expt. no.	Substrate	Oxygen uptake (μL/hr): Mitochondria	Mitochondria + supernatant
1	Succinate	45	167
	Citrate	53	221
	None	..	3
2	Citrate	57	226
	None	..	0
3	Citrate	49	121
	None	..	42

Each flask contained 0.5 ml mitochondria suspension, 10 micromoles $MgSO_4$; 14 micromoles AMP; 0.1 micromole cytochrome c and 30 micromoles succinate or citrate in 0.4 M sucrose + 0.02 M phosphate buffer, pH 7.0. The flasks with citrate contained, in addition, 1.2 micromoles CoA; 1.8 micromoles DPN^+; 0.9 micromole TPN^+ and 1.6 micromoles TPP. Total volume, 3.0 ml.

Mitochondria were isolated from 9-day-old seedlings; the seeds had not been stratified. The supernatant solution was that fraction remaining after removal of mitochondria from homogenates of embryos from unstratified, ungerminated seeds. All seeds were soaked in aerated water at 5° C for six hours. One ml of supernatant solution was added where indicated.

flavin adenine dinucleotide) had no effect. The addition of a liver coenzyme concentrate was also without an effect. It should be pointed out that the seedlings employed in the experiment described in table VII were 9 to 10 days old. The older age of the seedlings accounted for the relatively low activity observed.

Discussion

The experimental results reported here indicate that mitochondria of the Gymnosperm species, *Pinus lamberitiana*, contain the enzyme complex for oxidation of intermediates of the Krebs cycle. The properties of these preparations are similar to those reported for many other types of plant and animal tissues. The fact that pine embryo and seedling mitochondria possess enzymatic mechanisms for the oxidation of alanine and fatty acids further extends the likeness between pine mitochondria and those of other plants and animals (8).

Both microsomal and mitochondrial particles from sugar pine seedlings were found to oxidize palmitate-1-C^{14}. Studies on peanut mitochondria and microsomes have established the existence of a β-oxidation mechanism for fatty acids in the mitochondria and an atypical fatty-acid oxidase in the microsomes (25). The most reasonable interpretation of results obtained with pine seedling mitochondria and microsomes is that corresponding enzyme systems exist in the pine particles. However, further work including studies with palmitic acid labeled in carbon atoms other than C-1 is required to establish this point.

Several hypotheses may be offered to explain why the activity of the mitochondrial preparations from pine seeds decrease upon germination or stratification: first, enzymes localized in pine seed mitochondria may decrease during stratification and germination. Brummond and Burris (5) showed that an apparent change in the localization of malic and succinic dehydrogenases occurred in the mitochondria of maturing lupine plants. Second, the fragility of the particulates may increase with germination. Third, a metabolic inhibitor may accumulate. Fourth, the quantity of proteins or nitrogen containing macromolecules (nucleic acid) sedimenting with the mitochondria may increase with germination. This would cause an apparent decrease in the $Q_{O_2}(N)$. Finally, the concentration of an essential cofactor may decrease owing, perhaps, to its being catabolized or redistributed throughout the cell during germination or stratification. Some evidence for such a factor in non-stratified, ungerminated seed has been presented (table VII).

One purpose of this study was to gain greater insight into the germination process of sugar pine seed. It has been observed that although the pine seed is saturated with water, a lag occurs in active germination as evidenced by growth and respiration measurements (11, 23). This study has shown that the Krebs cycle enzyme complex is present in ungerminated sugar pine seed. Vanecko (27) has also detected Krebs cycle activity in mitochondria extracted from water saturated but ungerminated pea seeds. It is possible that the intial lag in germination of sugar pine seed is due to a lack of enzymatic cofactors. The lag could also result from a deficiency of substrates of the Krebs cycle or other enzymes. Bach (3) found all the dehydrogenases present in pea seeds, but concluded that the cofactors were essentially absent in the ungerminated seeds. DPN^+ has been observed to increase in wheat and bean seeds during the first four days of germination (22). Increases in the unsaturated fatty acids and invert sugars have been reported by Mirov during stratification of pine seeds (20).

Summary

1. Particulate cellular fractions were separated from seedlings and embryos of sugar pine (*Pinus lambertiana*). The particles centrifuging between 2,000 and $20{,}000 \times g$ catalyzed the oxidation of acids of the Krebs cycle. The cofactor requirements, and effects of malonate on these oxidations indicated that the particles are similar to mitochondria isolated from other plants and animals. This is the first such report for Gymnosperm species.

2. Pyruvate-2-C^{14} and acetate-2-C^{14} were metabolized by mitochondria from sugar pine. Malic, citric, and succinic acids isolated from the reaction mixture were radioactive.

3. Alanine-1-C^{14} and alanine-3-C^{14} were metabolized by the mitochondria of sugar pine and radioactive CO_2 was produced. Pyruvic, citric, and succinic acids isolated from the reaction mixture were radioactive.

4. The metabolism of palmitate-1-C^{14} and butyrate-1-C^{14} by pine mitochondria and microsomes indicated the presence of mechanisms for the oxidation of fatty acids.

5. The mitochondria from the embryos of ungerminated pine seeds showed a higher oxidative activity on various Krebs cycle substrates than particles from 5-day-old germinated seedlings, or particles from embryos of seeds stratified 60 or 120 days.

6. The mitochondria which showed decreasing oxidative activity with seedling development were stimulated by the supernatant from ungerminated seeds from which the mitochondria were removed. The stimulating factor was not present in the microsomes or the ash; it was stable to heat, unstable to acid and alkali, and not replaceable by flavin nucleotides.

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RESPIRATION AND THE OXIDATIVE ACTIVITY OF PARTICULATE FRACTIONS FROM DEVELOPING PEPPER FRUITS (CAPSICUM ANNUUM L.) [1,2]

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Organized respiratory activity has been associated with mitochondria isolated from various plant tissues, and it is now generally believed that mitochondria represent a major site of respiratory activity (8). Fruits have been used in relatively few mitochondrial investigations, and only the work of Millerd et al (10) dealt with the relative actvities of certain particulate fractions in relation to fruit development.

The present study was undertaken to investigate the oxidative activity of particulate fractions from developing pepper fruits and to compare this activity with that of the intact tissue.

[1] Received April 2, 1957.

[2] The data reported here are taken from the Ph.D. thesis of F. D. Howard, presented to the graduate division of the University of California at Berkeley, January, 1957.

Materials and Methods

Fruits from pepper plants (*Capsicum annuum* L., var. California Wonder) field-grown at Davis, California, were used. Freshly harvested fruits were taken to the laboratory and sorted for specific stages of development (maturity) after which samples were prepared for subsequent analyses.

ATP (adenosine triphosphate), CoA (Coenzyme A), DPN (coenzyme I), and cocarboxylase were purchased from Nutritional Biochemicals Corporation. EDTA (ethylenediamine tetraacetic acid) was donated by the Geigy Company, Inc.

Buffer solutions used had the following composition:

Buffer I —0.10 M phosphate buffer (KH_2PO_4, Na_2HPO_4) pH 6.8, 0.40 M sucrose, 0.01 M EDTA;

Buffer II —0.01 M phosphate buffer pH 6.8, 0.40 M sucrose;

Buffer III—0.02 M phosphate buffer pH 6.8, 0.30 M sucrose, 0.02 M glucose, 0.002 M $MgSO_4$.

Selection of Maturity Groups: For this study, fruits representative of stages of development varying from immature green to full ripe were chosen. The maturities selected will be referred to as immature greens, mature greens, breakers, half ripes, and full ripes. Immature greens were most difficult to select, since no adequate method was known for selecting fruits of the same physiological age. Fruits of the same chronological age may differ in physiological age because of differences in growth rate. Fruit weight (less than 100 grams) and appearance of the surface were used in selecting immature greens. In tests the seeds from these fruits failed to germinate. Mature greens were selected on the basis of size, color (all green), and ability of seeds to germinate. Other maturities were selected according to size and amount of red color visible on the surface. Breakers were up to 25 % red, half ripes were 50 % red, and full ripes were all red. Approximately 4 to 6 weeks are required for fruits to develop from blossom to mature greens, and another 3 to 4 weeks are required for fruits to become full red. Temperature is an important factor in the rate of fruit development. The time required for a fruit to develop is quite unpredictable as Gustafson (3) has pointed out for tomatoes. Thus, chronological age is of doubtful value in selecting a given maturity.

Tissue Slice Respiration: Respiratory responses were measured by the indirect Warburg technique described by Umbreit et al (14). This was used for measuring both CO_2 production and O_2 uptake of tissue slices.

Tissue slices, representing tissue from the stem to blossom ends, were selected from material representing all stages of maturity. The fruits were harvested and the sections were immediately cut and weighed. Duplicate 25-gram samples were placed into 100-ml vessels and allowed to come to equilibrium in the dark at 25° C. The time-of-harvest was arbitrarily used as the zero time and only the 4.5 to 5.5-hour readings are reported, since it was found that the respiratory rate rises to this point and then decreases. Both O_2 uptake and CO_2 production were determined.

Preparation of the Particulates: The cell particulates were isolated from fruit wall tissue, using a method similar in principle to that described by Millerd et al (9) as modified by Price and Thimann (12). The procedure was also modified to include EDTA in Buffer I as suggested by Lieberman and Biale (7).

Forty fruits were selected for each immature green sample; ten fruits were selected for each sample of other maturities. The fruits in a sample were halved, and thin slices were cut from stem to blossom end, using only the fruit wall tissue (placenta and septal tissues were removed). Two 100-gram aliquots were placed in tared beakers in a forced-draft oven at 70° C for dry weight. These were subsequently analyzed for protein nitrogen according to a micromethod described by Clark (2). Two additional 100-gram aliquots were separately chopped into a micro Waring blendor jar containing 100 ml of buffer I and blended for 2 minutes at about 5000 rpm. The resulting slurry was filtered through 4 layers of cheesecloth into a 400-ml beaker, and the pulp re-extracted with 50 ml of buffer I. The filtrate was centrifuged at $500 \times g$ (Servall SS-I) for 5 minutes to remove cellular debris (ppt-I). The supernatant (S-I) was decanted and centrifuged at $16{,}000 \times g$ for 15 minutes. The supernatant (S-II) from the second centrifugation was decanted, and all precipitates (ppt-II) were resuspended in one centrifuge tube with 40 ml of buffer II. This material was again centrifuged at $16{,}000 \times g$ for 15 minutes, producing supernatant (S-III) and precipitate (ppt-III). Aliquots totaling 10 ml of buffer III were used to transfer ppt-III to a Potter-Elvejhem homogenizer. This mixture, homogenized for approximately 20 seconds in an ice-water bath, will be referred to as the particulates or ppt-III. Freshly prepared particulate suspensions were used without further dilution. A 0.5-ml aliquot (containing approx. 0.5 mg protein nitrogen and equivalent to about 10 grams fresh weight) was used in each vessel for all O_2 uptake measurements.

Oxygen Uptake Experiments: Respiratory activity of particulates was measured by oxygen uptake using a Warburg respirometer and conventional methods (14). Appropriate cofactors and substrates, dissolved in buffer III and adjusted to pH 6.8, were pipetted into the main body of the Warburg vessels. The particulate suspension was then pipetted either into the sidearm or directly into the reaction mixture as described by Humphreys and Conn (4). The center well contained a small piece of folded filter paper to which was added 0.1 ml of 5 *N* NaOH. The final volume of the reaction mixture was 1.9 ml unless otherwise stated. O_2 uptake is reported as microliters per hour per mg N (Q_{O_2} (N)). The nitrogen content of the particulate suspension is shown in appropriate tables.

Results

Fractional Distribution of Protein Nitrogen: The distribution of protein nitrogen among the various fractions isolated during the preparation of particulates from fruit wall tissue is shown in table I. Duplicate tissue samples were selected. One was used for the determination of the protein nitrogen content of fruit wall tissue. The other was fractionated, as previously described, for the isolation of the enzyme extract, and the total protein nitrogen content of each fraction was determined. Data on the fractions isolated from the fruit wall tissue of four stages of immature greens, mature greens, breakers, and full ripes are shown in table I.

The protein nitrogen content of the fruit wall tissue decreased rapidly as the immature greens increased in size from 10 to 15 grams to 30 to 40 grams and then with some variability showed a considerable decrease between 60 to 80 grams and 100 to 180 grams. This is reflected in the respiration curves when the values are expressed on a nitrogen basis.

The protein nitrogen content of the fractions obtained from fruit wall tissue was variously distributed. The pulp from immature greens contained 17.1 to 22.4 % of the total protein nitrogen content of the fruit wall tissue, 22.2 % from mature greens, and 10.7 to 17.0 % from more mature fruits. The pulp contained material which was washed once with 50 ml of buffer I and did not pass through the four layers of cheesecloth. Ppt-I contained from 2.5 to 7.1 % of the total protein nitrogen. The data indicate a gradual decrease in the nitrogen content of this fraction with maturity. Ppt-I contained insoluble matter which precipitated at $500 \times g$, such as nuclei, plastids, and large cellular debris. Supernatant II contained 66 to 80 % of the protein nitrogen. S-II contained both soluble and insoluble components of the cytoplasm which did not precipitate when centrifuged at $16{,}000 \times g$. S-III which resulted from washing ppt-II with 40 ml of buffer II contained from 1.0 to 2.7 % of the protein nitrogen. Ppt-III contained from 3.1 to 5.3 % of the total protein nitrogen content. On a fresh-weight basis, the nitrogen content of the particulate fraction decreased as the fruit approached senescence. Ppt-III was the fraction used to study the particulate activity of developing fruits shown below.

Respiratory Response of Tissue Slices from Developing Pepper Fruits: The oxygen uptake of tissue slices as related to age in days for the immature greens, and size and color for the other maturities, is shown in fig 1. The data are expressed on both a fresh-weight and nitrogen bases. On the fresh-weight basis, O_2 uptake decreased markedly with an increase in age of the fruit. On a nitrogen basis, this decrease was not as marked, due to the higher nitrogen content of the younger and smaller fruits. As the fruits attained full size, the O_2 uptake remained relatively steady and then declined after the fruits became fully mature. Similar results were obtained for immature fruits when fruit weight, rather than age in days, was used as an index of maturity.

The physiological age of the fruits selected to represent a given maturity was quite variable. It was

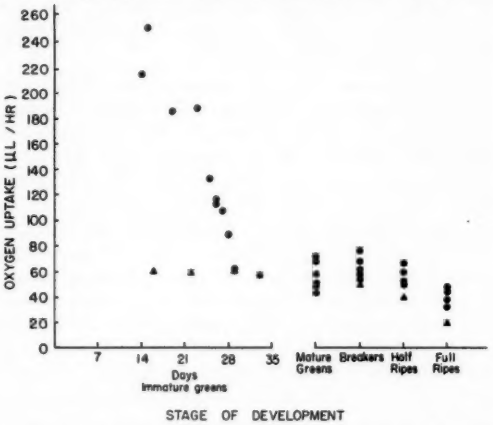

Fig. 1. Effect of maturity on oxygen uptake by pepper fruits at various stages of maturity. For immature green fruits, maturity is indicated by age in days from flowering; other maturities are denoted by color change. Determinations were made at 25° C. ● represents values expressed on a F.W. basis; ▲ represents values expressed on a nitrogen basis.

Table I

Distribution of Protein Nitrogen in Fractions Separated During the Isolation of Particulates from Various Maturities

Maturity	Fruit wt (gm)	Protein-N gm/100 gm F.W.	Percent in each fraction				
			Pulp	Ppt-I	S-II	S-III	Ppt-III
Immature greens	10-15	0.27	17.1	7.1	68.0	2.7	5.3
	30-40	0.19	22.4	5.1	70.0	1.5	4.4
	40-60	0.21	21.4	4.4	67.0	1.7	4.6
	60-80	0.23	...	4.6	76.0	2.5	5.3
Mature greens	100-180	0.16	22.2	3.9	66.0	1.0	4.7
Breakers	180-250	0.17	10.7	2.9	80.0	2.4	4.0
Full ripes	180-250	0.19	17.0	2.5	75.0	2.1	3.1

TABLE II

RESPIRATORY RATES OF TISSUE SLICES FROM DEVELOPING PEPPER FRUITS

MATURITY	FRESH WT BASIS μGM/HR × GM F.W. **		NITROGEN BASIS * μL/HR × MG N **		R.Q.
	O_2 UPTAKE	CO_2 OUTPUT	O_2 UPTAKE	CO_2 OUTPUT	
Immature greens	116	100	65	56	0.86
Mature greens	88	71	51	41	0.81
Breakers	73	61	42	36	0.84
Half ripes	82	66	49	39	0.81
Full ripes	46	38	23	18	0.83

* Calculated on the basis of the protein nitrogen content of the same tissue used for the respiration measurements.

** Based on readings taken 4.5 to 5.5 hours after harvest.

therefore difficult to connect the points on the curve and come to any conclusion regarding a climacteric although, superficially, it appears to be present. In experiments where a climacteric has been demonstrated (5, 6), whole fruits were harvested and measurements made throughout the ripening period.

O_2 uptake and CO_2 production from immature greens, mature greens, breakers, half ripes, and full ripes were compared on both a fresh-weight and a nitrogen basis (table II). On a fresh-weight basis, O_2 uptake and CO_2 production from immature green fruit were 24 to 29 % greater than from mature greens, and even greater differences existed between immature greens and other maturities. The numerical differences were not nearly so large on a nitrogen basis; however, immature green fruits showed rates about 25 % greater than those from mature greens. Since CO_2 production was less than O_2 uptake in all cases, the R.Q. was less than unity. Essentially no difference in R.Q. was obtained with increasing maturity. After 24 hours in Warburg vessels at 25° C in the dark, R.Q. values were 0.91, 0.81, 0.95, 0.94, and 0.89, respectively, for immature green, mature green, breaker, half ripe, and full ripe. The values for breakers and half ripes are in good agreement with those obtained by Platenius (11), who reported an R.Q. of 0.96 for peppers after 24 hours of storage.

Expressing the data on a protein nitrogen basis rather than fresh-weight basis is sounder because of the difference in cell size between immature green fruits and other maturities, and because of the high and variable moisture content of the fruits. Using a protein nitrogen basis, the assumption is made that all of the protein of the tissue is involved in the respiratory process. However, such an assumption is not entirely valid. Regardless of the basis used to express the data, immature green fruits exhibit greater respiratory rates than fruits of other maturities.

If the activity of the particulate fraction contributes significantly to the respiration of intact fruit, a decrease in the nitrogen content with maturity might explain, in part, the decreased respiratory response observed with the more mature fruits.

OXIDATIVE ACTIVITY OF PARTICULATE FRACTIONS FROM DEVELOPING PEPPER FRUITS: Because it has been postulated that mitochondria play a central role as controlling agents for the metabolic processes in the cell (8), the activity of this fraction in relation to maturity was investigated. Studies on particulates were carried out in detail on immature green and mature green fruits. The particulates were intended to represent the mitochondrial fraction.

The oxidation of malate by particulates from green fruits representative of various weight groups was studied. Depending upon size and maturity, each sample contained from 10 to 40 fruits which were similar.

In 20 determinations on samples of fruits which averaged less than 80 grams, the rate of malate oxidation varied from 0 to 16 $\mu l/hr \times mg$ N. On mature greens (100 to 205 gm fruits), the rate of malate oxidation was found to vary from 91 to 190 $\mu l/hr \times mg$ N. There was no agreement between fruit weight and activity, since different samples from fruits of the same average weight varied in their ability to oxidize malate as much as the samples from fruits of different average weights. For example, in two samples averaging 200 gm/fruit, the rate of malate oxidation varied from 105 to 190 $\mu l/hr \times mg$ N. From samples with average fruit weights of 98, 120, 150, 160, 175, and 180 the following rates were obtained: 168, 155, 96, 164, 91, and 140 $\mu l/hr \times mg$ N respectively.

The activity of pepper particulates with other Krebs intermediates was investigated. Citrate, isocitrate, succinate, fumarate and α-ketoglutarate were used. For the oxidation of α-ketoglutarate, malonate was added. Typical data are shown in table III. Oxidative activity is expressed in terms of the nitrogen content of the particulate suspension.

TABLE III

OXIDATION OF KREBS CYCLE INTERMEDIATES BY PARTICULATES ISOLATED FROM IMMATURE GREEN AND MATURE GREEN PEPPER FRUITS

SUBSTRATE	CONCENTRATION, M	Q_{O_2} (N)	
		IMMATURE GREENS	MATURE GREENS
Citrate	0.01	7	195
Isocitrate	0.01	6	125
Succinate	0.01	8	254
Fumarate	0.01	4	242
α-Ketoglutarate	0.02	1	94

The complete system contained 0.5 ml particulate suspension (1.33 mg protein N for immature greens isolated from 400 gm fruit wall tissue, 0.53 mg protein N for mature greens) 0.02 M phosphate buffer (pH 6.8), 0.3 M sucrose, 0.02 M glucose, 2×10^{-3} M $MgSO_4$, 5×10^{-4} M DPN, 10^{-3} M ATP, 9.3×10^{-5} M CoA, and 1.68×10^{-5} M cocarboxylase. In addition to these components, the system with α-ketoglutarate also contained 0.01 M malonate. The final volume of the reaction mixture was 1.4 ml.

When particulates from immature fruits were added to those from mature fruits a 15 % stimulation of succinate oxidation was observed indicating that no inhibiting substance was present and that the immature particulates may have contributed certain factors. Further, additions of boiled supernatant (S-II) from either immature or mature fruits did not significantly alter the oxidation rate of malate by immature or mature green particulates. The results further indicate that particulates from immature fruits were incapable of significantly oxidizing typical Krebs cycle intermediates, while mature green particulates readily oxidized them.

Since the protein nitrogen content of ppt-III from immature greens was as high or higher than that of similar preparations from other maturities, and further, the respiratory rates of intact tissues were greater on a fresh-weight and were comparable on a nitrogen basis, it would be expected that fruits weighing less than 100 grams would yield an active particulate preparation. However, as shown above, the particulates from immature fruits were not active.

In an attempt to eliminate the possibility of losses during isolation, immature green and mature green particulates were simultaneously extracted using the same reagents and identical conditions. Results similar to those reported above were obtained. It appears that immature green particulates lack some factor or factors which are associated with mature particulate preparations.

Cofactor Requirements: Table IV shows the effect of various concentrations of DPN and ATP on malate oxidation by mature green particulates. The data indicate that DPN becomes limiting when present at a final molarity between 1.2×10^{-4} and 2.0×10^{-4} M. ATP was found limiting at a final molarity between 2.0×10^{-4} and 3.0×10^{-4} M. In subsequent experiments, 5.0×10^{-4} M DPN and 1.0×10^{-3} M ATP were used.

Table IV

Effect of Various Concentrations of DPN and ATP on Malate Oxidation by Mature Green Particulates

DPN final molarity $\times 10^4$	ATP final molarity $\times 10^4$	Q_{O_2} (N)
0.2	10	33
0.4	10	69
1.2	10	82
2.0	10	104
2.8	10	90
3.6	10	110
5.0	10	91
2.0	1	90
2.0	2	98
2.0	3	118
2.0	4	121
2.0	5	114
2.0	6	114
2.0	8	108

The complete system contained 0.5 ml particulate suspension (0.51 mg protein N; equivalent to approximately 10 gm F.W.) 0.02 M phosphate buffer (pH 6.8), 0.3 M sucrose, 0.02 M glucose, 2×10^{-3} M $MgSO_4$. The final volume of the reaction mixture was 1.9 ml.

Table V

Effect of DPN, ATP, CoA, and Cocarboxylase Additions on the Oxidation of Malate by Immature Green and Mature Green Particulates

Conditions	Q_{O_2} (N)	
	Immature greens	Mature greens
Malate	2	16
Malate plus ATP	2	40
Malate plus DPN	0	58
Malate plus ATP and DPN	3	96
Malate plus CoA and cocarboxylase	7	0
Malate plus ATP, DPN, and CoA	10	125
Malate plus ATP, DPN, CoA, and cocarboxylase	16	167

The complete system contained 0.5 ml particulate suspension (0.565 mg protein N for immature greens; 0.43 mg protein N for mature greens), 0.02 M phosphate buffer (pH 6.8), 0.3 M sucrose, 0.02 M glucose, 2×10^{-3} M $MgSO_4$. Where added, the following concentrations of cofactors were used: 5×10^{-4} M DPN, 10^{-3} M ATP, 6.85×10^{-5} M CoA, and 1.23×10^{-4} M cocarboxylase. The final volume of the reaction mixture was 1.9 ml.

In the presence of DPN and ATP, the effect of CoA and cocarboxylase was studied. Beevers and Walker (1) demonstrated that in addition to DPN and ATP, CoA and cocarboxylase were required by castor bean mitochondria. They found that the addition of these cofactors stimulated different enzyme preparations from 100 to 700 % of the DPN-ATP established rates.

Table V presents typical data showing the effect of various cofactor combinations on malate oxidation by immature green and mature green particulates. In immature greens the magnitude of response to any of the combinations of cofactors was quite small and at best compares with malate oxidation by mature greens in the absence of any of these cofactors. Mature greens, on the other hand, showed a 200 % stimulation by the complete system over the DPN-ATP rate. CoA and cocarboxylase, in addition to DPN and ATP, are necessary cofactors for mature green particulates.

Discussion

Millerd et al (10) found that mitochondria from unripe avocado fruit (preclimacteric) were not inferior to those of ripe fruit (climacteric) in their capacity to oxidize substrates in systems containing adequate adenylate. The unripe avocado fruits were full size at harvest and may have already advanced through stages of development similar to those of mature green pepper fruits. However, it is not known whether particulates of other fruits exhibit the same characteristics as those of pepper fruits.

The observations do not explain the high respiratory rates obtained from immature fruits, but do indicate that there might be a different sequence of

events for immature, as compared with mature fruits. Since in vivo interpretations are difficult to make from in vitro data, one can only speculate concerning the events as they occur in the intact cell. One possibility concerning immature pepper fruits is that the cytoplasm contributes more than the particulate fraction in catalyzing the oxidation of Krebs cycle intermediates. Calculations made from the data indicate that the activity of the particulate fraction of immature fruits accounts for less than 5 % of the O_2 uptake by the intact tissue. On the basis of values obtained during succinate oxidation (table III) and data from figure 1 on the O_2 uptake from mature green fruits, the activity of mature green particulates accounts for about 45 % that of intact tissue. This may be explained by losses during isolation; however, the particulates were re-extracted as suggested by Price and Thimann (12), who obtained quantitative recoveries of succinic oxidase activity. It is possible that the cytoplasm makes a significant contribution, and such a hypothesis is in agreement with observations by Schneider and Hogeboom (13) concerning numerous in vitro experiments in which cell fractions had been combined variously. It is conceivable that with the advent of maturity, the summation of the cooperative efforts of cytoplasmic components declines as functional mitochondrial units develop.

Summary

The distribution of protein nitrogen among the various fractions isolated during the preparation of particulates was studied. The tissue used for this study represented seven stages of development from immature green to full ripe pepper fruits. From 66 to 80 % of the protein nitrogen was found in the supernatant after centrifugation at 16,000 × g, which presumably represents largely the soluble cytoplasmic fraction. The particulate fraction used for the enzyme studies contained from 3.1 to 5.3 % of the total protein nitrogen of the intact tissue and in general decreased with increasing maturity.

The respiratory rate of intact pepper fruit tissue declined with maturity. On a fresh-weight basis, immature fruits took up oxygen at a rapid rate. The rate of oxygen uptake rapidly decreases until the mature green stage was reached; then, after a slight rise it declined as the fruit approached senescence.

Particulates isolated from mature fruits yielded particulate suspensions which readily oxidized citrate, isocitrate, α-ketoglutarate, succinate, fumarate, and malate. CoA and cocarboxylase were required for maximal activity in addition to DPN, ATP, and Mg^{++}. Those isolated from immature green fruits were unable to significantly oxidize Krebs cycle intermediates with all of the cofactors used.

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HYDROGEN TRANSPORT AND OXIDATIVE PHOSPHORYLATION BY PARTICULATES FROM DEVELOPING PEPPER FRUITS[1,2]

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In a study of respiration and oxidative activity of particulates isolated from pepper fruits (11) it was shown that particulates from mature fruits oxidized various Krebs cycle intermediates and responded to additional cofactors, while those from immature fruits did not. Additions of coenzyme A and cocarboxylase to particulates from mature green fruits greatly stimulated oxygen uptake but produced only a very slight increase in preparations from immature fruits. Conceivably, this inactivity in particles from immature fruits may be due to lack of other factors.

This paper presents evidence that particulates from immature and mature green pepper fruits differ markedly in dehydrogenase activity and in their ability to transport hydrogen atoms and electrons from substrate and DPNH to oxygen. These differences could be reflected by phosphorus uptake; therefore, measurements on oxidative phosphorylation were made.

MATERIALS AND METHODS

Particulates were prepared from fruit wall tissue (pericarp) of developing pepper fruits as described previously (11).

DIP (2,6-dichlorophenolindophenol) was purchased from Eastman Organic Chemicals, Inc.; ATP (adenosine triphosphate), CoA (coenzyme A), DPN (coenzyme I), FAD (flavin adenine dinucleotide), and cocarboxylase from Nutritional Biochemicals Corporation; DPNH (reduced coenzyme I) and cytochrome c from Sigma Chemical Company. MB (methylene blue) was obtained from the National Aniline Division.

Dehydrogenase determinations were made by using frozen particulate suspensions (12) diluted approximately 25-fold. The absolute dilution factor depended upon the turbidity of the resultant solution and its effect on the colorimeter readings. This was adjusted so that readings on suspension blanks were between 95 and 100 % transmission.

Measurements of dehydrogenase activity were made in Thunberg tubes by determining the rate of anaerobic reduction of DIP (16, 18), using an Evelyn colorimeter with a number 600 filter. This filter gave the greatest sensitivity for the dye at pH 7.0, with only a slight shift in the peaks at other pH values. The reaction mixture contained 0.02 M substrate, 0.062 M phosphate buffer (pH as indicated) 0.002 M $MgSO_4$, 5×10^{-5} M DIP, 0.001 M DPN and particulates (approximately 0.02 mg protein nitrogen), in a final volume of 8.0 ml.

In order to convert optical density values to oxygen uptake, the redox equivalent of 10^{-5} M DIP was determined. It was found, by reducing 5 ml of 10^{-5} M DIP with an equivalent amount of ascorbic acid, that a unit change in optical density corresponded to 0.585 micromoles (1.17 microequivalents) of dye. Since one microequivalent is equal to 5.6 microliters of O_2 under standard conditions and a unit of optical density is equal to 1.17 microequivalents, it follows that each unit of optical density corresponds to 6.55 microliters of oxygen taken up.

The oxidation of malate, succinate, and DPNH was followed in Warburg vessels, using methods previously described (11).

The oxidation of reduced cytochrome c was followed in a Beckman spectrophotometer (model DU), modified with a photomultiplier circuit similar to that described by Brown et al (2). These experiments were conducted with frozen particulates at room temperature, and the measurements of optical density were made at a wave length of 550 $m\mu$. The reaction mixture had the following initial composition: 1.33×10^{-5} M cytochrome c, 5.3×10^{-4} M sodium hydrosulfite, 0.02 M phosphate buffer pH 6.8, 0.3 M sucrose, 0.02 M glucose, 0.002 M $MgSO_4$ and 0.1 ml particulate suspension (0.01 mg to 0.02 mg protein nitrogen) in a final volume of 3.0 ml.

Initial readings were taken, then the particulate suspension was added and the percent transmission of light was recorded with time. Particulates were omitted to determine auto-oxidation of cytochrome c. Turbidity, due to the particulates, was compensated by blanks.

Phosphorus was determined by using the method of Fiske and SubbaRow (5). Protein nitrogen was determined by a micromethod described by Clark (3).

RESULTS AND DISCUSSION

DEHYDROGENASE ACTIVITY: Succinate, malate, and isocitrate dehydrogenase activities associated with particulates isolated from immature and mature green fruits were determined at various pH values (table I). DIP was used rather than MB, since it has been shown that the latter strongly inhibits succinate dehydrogenase activity of oat and pea particulates (16).

The data indicate that immature green pepper particulates have less succinate, malate, and isocitrate dehydrogenase activities than mature particulates. Isocitrate dehydrogenase activity of the immature particulates was extremely small in comparison

[1] Received April 12, 1957.

[2] The data reported here are taken from the Ph.D. thesis of F. D. Howard, presented to the graduate division of the University of California at Berkeley, January 3, 1957.

TABLE I

SUCCINATE, MALATE, AND ISOCITRATE DEHYDROGENASE ACTIVITIES OF IMMATURE GREEN AND MATURE GREEN PEPPER PARTICULATES AT VARIOUS pH VALUES *

	RATE: Δ OD/HR × MG N					
	IMMATURE GREEN FRUITS			MATURE GREEN FRUITS		
pH	SUCCINATE	MALATE	ISOCITRATE	SUCCINATE	MALATE	ISOCITRATE
5.7	5.4 (± 0.6) **	.....	0.0	15.0 (± 1.0)	.....	.....
5.8	6.0 (± 0.6)	.....	..	.....	.....	.....
6.2	6.9 (± 0.7)	4.5 (± 0.5)	0.0	18.6 (± 1.0)	.....	3.3 (± 0.5)
6.5	6.3 (± 1.2)	7.2 (± 1.2)	0.0	18.0 (± 1.0)	9.6 (± 1.0)	.....
6.6	.....	.....	..	.....	.....	4.5 (± 0.5)
6.8	6.0 (± 1.0)	.....	0.0	.....	.....	6.0 (± 0.6)
6.9	0.2 (± 0.2)	.....	0.9 (± 0.15)	2.4 (± 0.1)	.....	3.6 (± 0.4)
7.2	0.1 (± 0.2)	6.9 (± 0.0)	0.3 (± 0.0)	.....	10.8 (± 1.0)	.....
7.3	0.0	.....	..	.....	.....	.....
7.4	.....	6.6 (± 0.2)	..	.....	11.4 (± 1.0)	.....
7.6	.....	6.3 (± 0.1)	0.0	.....	.....	0.6 (± 0.0)

* Particulate suspensions were diluted about 25-fold before use. The complete system contained 0.062 M phosphate buffer, at the desired pH, 0.02 M substrate, 2×10^{-3} M $MgSO_4$, 2.0 ml of diluted particulate suspension (approximately 0.05 mg protein N), 1×10^{-5} M DIP, and water to a final volume of 8.0 ml. For malate and isocitrate dehydrogenase measurements 1×10^{-3} M DPN was added. All readings were taken on Thunberg tubes under vacuum at 30° C, by using an Evelyn colorimeter with a No. 600 filter.

** Values in parenthesis represent deviations from the mean.

to that of the mature ones. Succinate dehydrogenase activity of the particulates in the range from pH 5.7 to 6.5 was about 33 % that of the nature. At pH 6.5 malate dehydrogenase activity of immature particulates was 75 % that of the mature greens.

These data suggest that in the condition in which they were obtained, particulates from immature fruits were not functional since at least one enzyme of the TCA cycle (isocitrate dehydrogenase) was relatively inactive. The low activity of this enzyme partly explains the slow oxidation of isocitrate from immature green pepper particulates reported previously (11). No evidence could be found in the literature concerning the lability of isocitrate dehydrogenase. However, there is evidence to indicate a solubilization effect during blending, which results in losses of DPNH-cytochrome c reductase and succinate dehydrogenase (9). Since the mature particulates show good dehydrogenase activity and were isolated under conditions identical to those used for the immature, it is possible that those enzymes that were either lacking or less active were not as yet associated with these particles.

THE EFFECT OF MB, FAD, AND CYTOCHROME C ON SUBSTRATE OXIDATION: The data in table II show that malate oxidation by immature particulates was increased upon the addition of MB, FAD, cytochrome c, or FAD plus cytochrome c. Although the rate of malate oxidation by immature particulates upon the addition of FAD and cytochrome c was increased over 500 %, still the O_2 uptake on a nitrogen basis was only about one-fifth that of the mature particulates. The rate of malate oxidation was probably limited by the amount of malate dehydrogenase associated with immature particulates, as well as by the amount of the cofactors added.

In the case of mature particulates, additions of MB, FAD, cytochrome c, and FAD plus cytochrome c gave variable responses. With MB or FAD, the observed rates were about 15 % lower than the established malate rate, while increases of 18 and 15 %, respectively, were observed with cytochrome c or FAD plus cytochrome c. It appears that immature particulate suspensions require the addition of FAD and cytochrome c to oxidize malate. Mature particulates, on the other hand, need only cytochrome c.

The effect of cytochrome c on succinate oxidation was also studied. Without cytochrome c the succinate oxidation rate by immature particles was 10 μl/hr × mg N. Upon adding 6.38×10^{-5} M cytochrome c, a

TABLE II

THE EFFECT OF MB, FAD, CYTOCHROME C AND FAD PLUS CYTOCHROME C ON MALATE OXIDATION BY IMMATURE GREEN AND MATURE GREEN PEPPER PARTICULATES *

COMPONENTS	Q_{O_2} (N)	
	IMMATURE GREENS	MATURE GREENS
Malate	6	128
Malate plus MB	30	107
Malate plus FAD	34	109
Malate plus cytochrome c	22	152
Malate plus FAD and cytochrome c	31	147

* The complete system contained 0.5 ml particulates suspension (0.87 mg protein N for immature greens; 0.65 mg proetin N for mature greens), 0.02 M phosphate buffer (pH 6.8), 0.3 M sucrose, 0.02 M glucose, 2×10^{-3} M $MgSO_4$, 5×10^{-4} DPN, 1×10^{-3} M ATP, 6.85×10^{-5} M CoA, 1.23×10^{-4} M cocarboxylase, and 0.01 M malate. Where added 5×10^{-3} M MB, 3.35×10^{-4} M FAD, and 6.38×10^{-5} M cytochrome c were used. The final volume of the reaction mixture was 1.9 ml.

rate of 79 μl/hr × mg N was obtained, while with mature particulates O_2 uptake was increased from 160 to 197 μl/hr × mg N.

Isocitrate was not oxidized by the immature particulates even with MB, FAD, or cytochrome c. These results were in agreement with the previous observation that isocitrate dehydrogenase activity of immature particulates was extremely low (11).

Oxidation of DPNH: DPNH was used as a substrate because of differences in dehydrogenase activities of immature and mature green particulates. Figure 1 shows the effect of MB, FAD, cytochrome c, and FAD plus cytochrome c on DPNH oxidation by immature particulates. The curves show that during the first 10-minute interval the effects of FAD and cytochrome c were additive. The O_2 uptake was essentially completed in 20 to 30 minutes. The magnitude of the reaction was limited, apparently by the supply of DPNH available to the system (14).

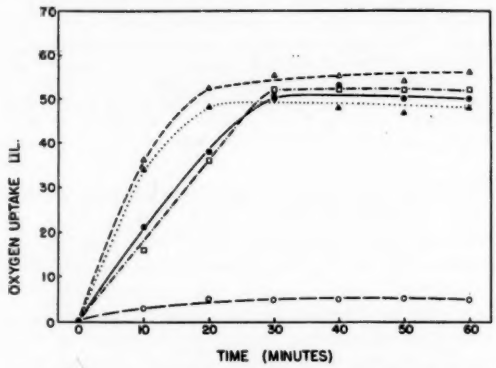

Fig. 1. The effect of MB, FAD, cytochrome c, and FAD plus cytochrome c on DPNH oxidation by particulates from immature green pepper fruiits. ○, enzyme plus DPNH; □, enzyme plus DPNH and FAD; ●, enzyme DPNH and cytochrome c; ▲, enzyme plus DPNH, FAD, and cytochrome c; △, enzyme plus DPNH and MB.

At low concentrations of DPNH (2.6×10^{-4} M), very little oxidation was observed; however, when DPNH was supplied at levels approaching those used for other substrates (5×10^{-3} M), then DPNH oxidation by mature particulates could be followed easily. Rates from 51 to 91 μl/hr × mg N were observed. On the other hand, when 5×10^{-3} M DPNH was supplied to a system containing immature green particulates, rates from 5 to 20 μl/hr × mg N were obtained. It was found that the addition of MB stimulated DPNH oxidation, and rates from 46 to 80 μl/hr × mg N were obtained with different immature green preparations.

DPNH was readily oxidized by mature particulates (fig 2). The addition of MB, FAD, cytochrome c, or FAD plus cytochrome c did not alter the magnitude of the reaction but these cofactors did affect the rate at which equilibrium was reached.

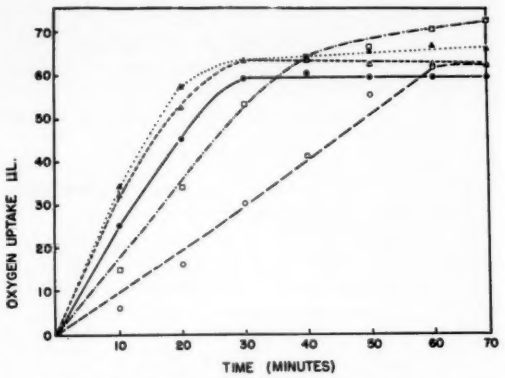

Fig. 2. The effect of MB, FAD, cytochrome c, and FAD plus cytochrome c on DPNH oxidation by particulates from mature green pepper fruits. ○, enzyme plus DPNH; □, enzyme plus DPNH and FAD; ●, enzyme plus DPNH and cytochrome c; ▲, enzyme plus DPNH, FAD, and cytochrome c; △, enzyme plus DPNH and MB.

These experiments show that particulates from the two maturities differed significantly in their respective ability to transport electrons to oxygen. DPNH oxidation by immature particulates was increased by FAD and cytochrome c but did not affect the magnitude of reaction in mature particulates. It would appear that these factors were absent from the particulates of immature fruits and it seems unlikely that they were removed or lost during the isolation procedure.

Oxidation of Reduced Cytochrome c: Cytochrome oxidase has been associated with mitochondria in both animal and plant cells (8). The presence of this enzyme on potato particulates has been established (7, 10). Figure 3 shows that both immature and mature green particulates were equally capable of oxidizing reduced cytochrome c. Cytochrome oxidase activity appears to be about equally associated with

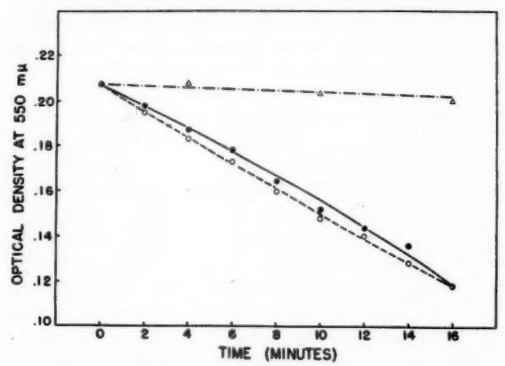

Fig. 3. Cytochrome oxidase activity of particulates isolated from immature green and mature green pepper fruits. ●, enzyme from immature greens; ○, enzyme from mature greens; △, blank.

particulates from both maturities. It is further indicated that cytochrome oxidase would probably not be a limiting factor in the activity of immature particulates.

P : O Ratios: With immature and mature particulates, P : O ratios of different Krebs cycle substrates were determined. Results obtained with mature particulates are shown in table III. Corresponding changes for immature particulates were so small that it was concluded that essentially no oxidative phosphorylation took place even with the addition of cytochrome c. The data show that mature particulates were capable of removing inorganic phosphate from the ambient solution during the course of substrate oxidation. P : O values ranging from 0.92 for succinate to 1.89 for isocitrate were obtained. These values in general compare favorably with those obtained by other investigators (1, 10, 13, 17). The values for α-ketoglutarate and succinate, however, are low in comparison to those recently obtained (4, 6, 15).

TABLE III

P:O RATIOS OF DIFFERENT KREBS CYCLE SUBSTRATES OBTAINED WITH MATURE GREEN PEPPER PARTICULATES

SUBSTRATE *	CONCENTRATION	OXYGEN UPTAKE **	PHOSPHATE UPTAKE **	P:O
Citrate	0.01 M	9.2	14.45	1.57
Isocitrate	0.01 M	5.9	11.15	1.89
Succinate	0.01 M	12.2	11.20	0.92
Fumarate	0.01 M	11.4	15.84	1.39
Malate	0.01 M	10.0	15.86	1.59
α-Ketoglutarate	0.02 M	4.5	7.93	1.76

* In addition to substrate the complete system contained 0.5 ml particulates suspension (0.53 mg protein N), 0.02 M phosphate buffer (pH 6.8), 0.3 M sucrose, 0.02 M glucose, 2×10^{-3} M $MgSO_4$, 5×10^{-4} M DPN, 10^{-3} M ATP, 9.3×10^{-5} M CoA, and 1.68×10^{-5} M cocarboxylase. In addition to these components the system with α-ketoglutarate also contained 0.01 M NaF and 0.01 M malonate. The reaction was stopped by the addition of 0.5 ml of 10% trichloroacrtic acid from the sidearm. Initial blanks were obtained by the addition of trichloroacetic acid at zero time to duplicate vessels. The final volume of the reaction mixture was 1.4 ml.

** Uptake in microatoms per 1.4 ml per hr.

SUMMARY

Succinate, malate, and isocitrate dehydrogenase activities associated with particulates isolated from immature and mature green pepper fruits were studied. Particulates from immature fruits had less succinate and malate dehydrogenase activity per unit of nitrogen than those from mature fruits. Very slight isocitrate dehydrogenase activity was found associated with immature particulates at pH values of 6.9 and 7.2; while that of mature particulates was quite high.

The DPNH oxidation by mature green pepper particulates was followed in Warburg vessels, with rates varying from 51 to 91 $\mu l/hr \times mg$ N. Particulates from immature fruits oxidized DPNH at rates of 5 to 20 $\mu l/hr \times mg$ N. The addition of FAD and/or cytochrome c stimulated DPNH as well as malate and succinate oxidation by immature particulates. Methylene blue could replace the FAD or cytochrome c requirement.

Cytochrome oxidase activity was found to be about equally associated with particulates from both immature and mature fruits.

Essentially no oxidative phosphorylation was observed in immature green pepper particulates. Particulates from mature fruits gave P : O values ranging from 0.92 for succinate to 1.89 for isocitrate.

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ABSORPTION AND MOBILITY OF FOLIAR APPLIED NUTRIENTS[1,2,3]

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One criterion of the effectiveness of nutritional sprays is the rate at which the foliar applied nutrients are absorbed by the leaf and translocated to other plant parts including the roots. Two distinct but not easily separated processes, absorption and transport, are involved. Initial penetration most likely involves passage through the cuticle and epidermal cells (17, 20); stomata may also play a minor role (15). Transport of foliar absorbed nutrients undoubtedly occurs via the phloem (2, 3, 4, 14). While the rate and amount of transport varies with each element, it parallels the phenomenon of nutrient redistribution as described by Biddulph (2) and Williams (19). The extent of redistribution (mobility, phloem transport) of each nutrient in the plant is an important consideration in foliar applications of fertilizer to satisfy nutrient needs of plants (20), in assaying the capacity of meristems to initiate "internal starvation" (19), and in "keying-out" nutritional deficiencies (2). Symptoms of malnutrition may arise from lack of mobility as well as lack of initial absorption.

Only in a few instances and with a limited number of the essential elements have rates of foliar absorption and transport been reported (2, 7, 14, 15, 20). As with herbicides (8, 17) isotopic labeling offers a unique approach to studies of nutrient absorption, transport (export) from leaves, and mobility. The rates of absorption, by the primary leaves of the bean, of several isotopically labeled nutrient elements, and their subsequent mobilities and partitioning within plant parts are herein presented.

Materials and Methods

A black seeded Blue Lake bean variety (Rogers Bros., Twin Falls, Idaho, Stock No. 42335) was selected as the experimental plant because of its uniformity. Seeds were germinated in coarse No. 8 quartz sand (American Graded Sand Co., Chicago). After emergence the seedlings were transferred to aerated solution cultures described by Asen, Wittwer and Teubner (1).

The radioactive isotopes, their physical and chemical characteristics, and the pH of the treating solutions used are listed in table I. Radioactive materials, both processed and irradiated units, were obtained from Oak Ridge National Laboratory, Oak Ridge, Tennessee, Nuclear Science and Engineering Corporation, Pittsburgh, Pennsylvania, or the Abbott Laboratories, North Chicago, Illinois. Solutions of each were prepared with the appropriate carrier at concentrations non-injurious to leaf surfaces, and at pH levels (table I) previously determined favorable for rapid uptake.

Table I

Physical and Chemical Characteristics of Isotopes and pH of Treating Solutions

Isotopes	Chemical form	Specific activity	pH of treating solutions
Na^{22}	NaCl	Carrier free	4.5
P^{32}	H_3PO_4	15173 mc/gm	3.0
S^{35}	SO_4	Carrier free	3.0
Cl^{36}	HCl	0.586 mc/gm	3.0
K^{42}	K_2CO_3	20 mc/gm	9.0
Ca^{45}	$CaCl_2$	102 mc/gm	3.0
Mn^{52-54}	$MnCl_2$	Carrier free	3.0
Fe^{55-59}	$FeCl_3$	2.60 mc/gm	2.0
Cu^{64}	$Cu(NO_3)_2$	4660 mc/gm	3.0
Zn^{65}	$ZnCl_2$	148 mc/gm	3.0
Rb^{86}	$RbCl_2$	576 mc/gm	3.0
Sr^{89}	$SrCl_2$	Carrier free	3.0
Mo^{99}	$(NH_4)_2MoO_4$	101 mc/gm	8.0
$BaLa^{140}$	$BaCl_2$	Carrier free	3.0

The isotopically labeled solutions were applied within 48 hours after the plants were transferred to the solution cultures. A small drop (0.01 ml) of the radioactive solution was placed in the center on the upper surface of one of the primary leaf blades at the stage of development illustrated in figure 1. Experimental variables known to have an effect on foliar absorption of nutrients as determined in previous tests were standardized (9, 10, 14, 15, 20). Plants were grown under the long (15 to 16 hours) days of late spring and early summer, temperatures of from 20 to 30° C were maintained and all isotopes were applied from 8 to 9 A.M.

At various intervals after treatment plants were harvested and partitioned as follows (fig 1). A circular disc (A) 1 cm in diameter, was first removed, which included the spot to which the radioactive solution was applied. The plant was then separated into (B) the remainder of the treated leaf, (C) stem plus non-treated leaf and (D) the roots.

[1] Received April 16, 1957.

[2] Journal Article No. 2059 from the Michigan Agricultural Experiment Station.

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In this paper, the term nutrient refers to what are now known as essential as well as some non-essential elements.

The plant parts were cut into small segments placed in 50-ml beakers, dried at 70° C in a forced draft oven and the dried samples assayed directly in the beakers for radioactivity using an end-window G-M tube and standard scaler circuit. Self-absorption was found negligible for all isotopes with the exception of S^{35} and Ca^{45}.

Absorption of each nutrient and its subsequent mobility and distribution was measured by determining the percent of applied isotope recovered in non-treated plant parts. This was calculated from the total radioactivity applied to each plant and the radioactivity of the individual parts. Percent absorption at a given interval after treating was calculated as the percent of the total radioactivity recovered in all plant parts shown in figure 1 exclusive of the leaf disc (A) which included the site of application.

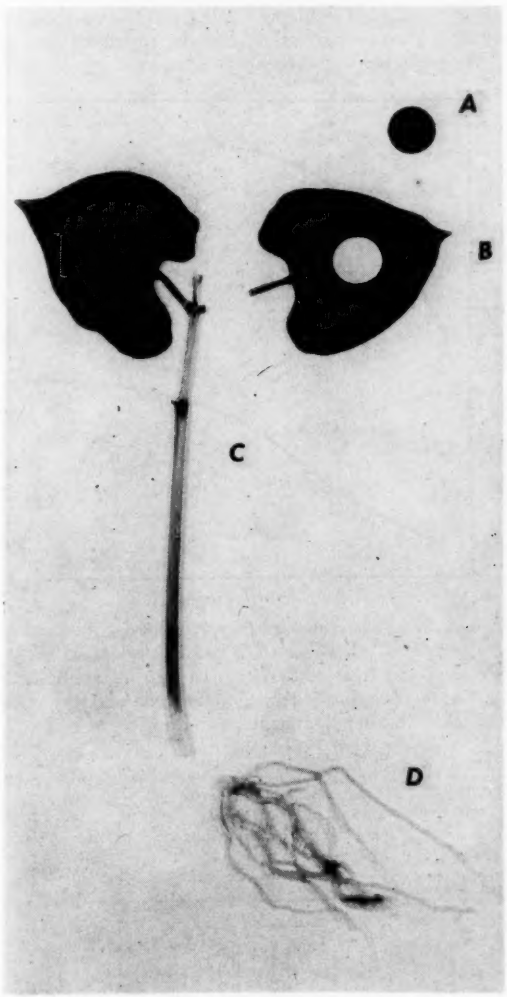

FIG. 1. Typical bean plant and parts assayed for radioactivity. A—One-cm diameter disc to which the isotope was applied, B—Remainder of treated leaf, C—Stem plus non-treated leaf, D—Roots.

It was not possible to distinguish between the absorbed isotope and that remaining on the surface of the 1 cm diameter leaf disc (fig 1-A). Therefore, the amount of each isotope absorbed at the site of application and translocated within the 1 cm leaf disc was not considered. When the entire leaf was immersed in the labeled solutions (table III), absorption of the applied nutrient was measured in terms of the percent recovered in non-treated plant parts. With both methods the absorption values were lower than the true value.

Varying the concentrations in the treating solution by as much as 10-fold for each isotope showed a highly positive correlation between the total amount of each isotope applied to the leaf and the total amount absorbed and translocated. Thus, in the tables and figures which follow, all values are expressed as percentages of the total applied.

Two techniques were used to evaluate absorption rate and relative mobility of each isotopically labeled element. The radioactivity of all plant parts was determined at intervals of 6, 24, 48, 96 and 192 hours (shorter intervals were used for the short lived isotopes, K^{42} and Mo^{99}) after application to the leaf blade. Gross autoradiography of plants 6 and 24 hours after treatment with the isotope was used to confirm the transport data obtained by direct counting and to indicate sites of accumulation.

For radioactive counting five replicates, of a single plant each, were assayed for each time interval after treating with each isotope. Autoradiograms of three plants both 6 and 24 hours subsequent to treatment, were prepared after the circular disc which included the site of application of the isotope was removed. The otherwise intact plant specimens were then dried, exposed to x-ray film and autoradiograms prepared according to methods outlined by Wittwer and Lundahl (21). The values from three separate experiments for each labeled nutrient (table I) were utilized in the tabular and graphic data which follow.

RESULTS AND DISCUSSION

Figures 2 and 3 confirm previous reports (2, 7, 14, 15, 20) that all mineral elements are probably absorbed by leaves, but at greatly varying rates. The absorption curves (figs 2 and 3) vary from almost linear to asymptotic, with half times ranging from 6 hours or less for sodium (Na^{22}) and rubidium (Rb^{86}) up to undetermined, but much longer time intervals for iron (Fe^{55-59}) and molybdenum (Mo^{99}) (figs 2-A, 2-D and 3). The four groupings in figure 2 show that for a particular series of elements absorption and transport rates vary, but are closely parallel.

The isotopes most rapidly absorbed by the bean leaf were Na^{22}, K^{42} and Rb^{86}. Rapid uptake occurred with both methods of leaf placement (table III), and was accompanied by transport resulting in

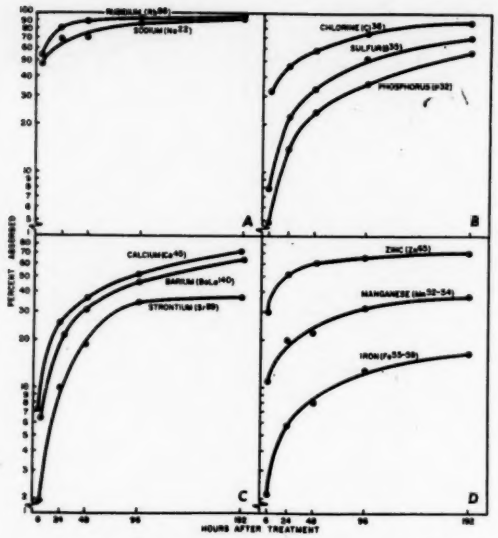

FIG. 2. Absorption–translocation curves illustrating the percent of the isotopes absorbed and translocated from the site of application on one of the primary leaves of the bean plant at 6, 24, 48, 96 and 192 hours after treatment. A—Rubidium (Rb^{86}) and sodium (Na^{22}); B—Chlorine (Cl^{36}), sulfur (S^{35}) and phosphorus (P^{32}); C—Calcium (Ca^{45}), barium ($BaLa^{140}$) and strontium (Sr^{89}); and D—Zinc (Zn^{65}), manganese (Mn^{52-54}) and iron (Fe^{55-59}).

an accumulation in the stems within 24 hours (tables II, III and fig 4), especially for Na^{22} and Rb^{86}.

Phosphorus (P^{32}) and sulfur (S^{35}) showed closely parallel absorption-transport rates (fig 2-B) with half times comparable to those already reported (7, 20). In agreement with the results of Biddulph (2) and Biddulph, Cory and Biddulph (3), transport and distribution of S^{35} compared favorably with P^{32}. More rapid leaf uptake occurred with S^{35} and Cl^{36} but their rates of export from the treated leaf were about the same as P^{32}. The usual metabolic gradient to meristems was apparent for both isotopes and is illustrated by the autoradiograms (figs 4-G and 4-H).

The distribution patterns for P^{32} and S^{35} within the bean plant are not easily distinguishable and resemble that of Zn^{65} (fig 5-B). In contrast, the anion Cl^{36} (fig 4-I) is distributed in a pattern similar to Na^{22}, K^{42} and Rb^{86} (figs 4-D, 4-E and 4-F).

That transport in the phloem may be a limiting factor in absorption and mobility of phosphorus, sulfur and chlorine is suggested by the data in tables II and III. Within 24 hours after application to the foliage high percentages (10 to 40) of the isotopes were found in the treated leaves with only minimum amounts (2 to 3 %) in the stem plus the non-treated leaf and roots. It has been suggested by Maizel, Benson and Tolbert (12), that phosphoryl choline, a constituent of plant saps, may act as a phosphorus carrier capable of penetrating cell membranes. Phloem transport of sulfur may be restricted if it is first complexed with organic constituents.

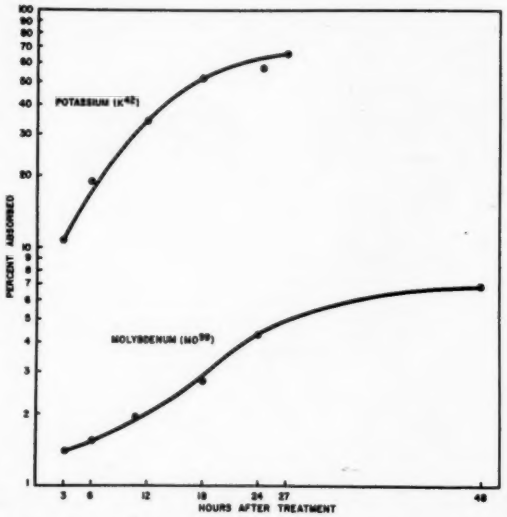

FIG. 3. Absorption–translocation curves illustrating the percent of potassium (K^{42}) and molybdenum (Mo^{99}) absorbed and translocated from the site of application on one of the primary leaves of the bean plant at various intervals after treatment.

TABLE II

ABSORPTION AND TRANSLOCATION OF FOLIAR APPLIED NUTRIENTS FROM SITE OF APPLICATION 24 HRS AFTER TREATMENT

NUTRIENT	ISOTOPE	TREATED LEAF	STEM + NON-TREATED LEAF	ROOT	TOTAL
		% total nutrient applied			
Phosphorus	P^{32}	10.5 ± 1.7 *	2.4 ± 0.3	1.1 ± 0.6	14.0 ± 1.4
Sulfur	S^{35}	20.1 ± 4.7	1.6 ± 0.7	1.1 ± 0.6	22.8 ± 4.7
Chlorine	Cl^{36}	43.6 ± 11.3	2.3 ± 0.7	0.7 ± 0.2	46.6 ± 11.7
Sodium	Na^{22}	41.4 ± 7.1	25.5 ± 8.1	1.1 ± 0.6	68.0 ± 10.3
Potassium	K^{42}	42.5 ± 2.4	9.9 ± 1.4	4.7 ± 0.7	57.1 ± 2.7
Rubidium	Rb^{86}	53.2 ± 4.8	18.4 ± 2.6	8.7 ± 4.9	80.3 ± 4.5

* Standard error.

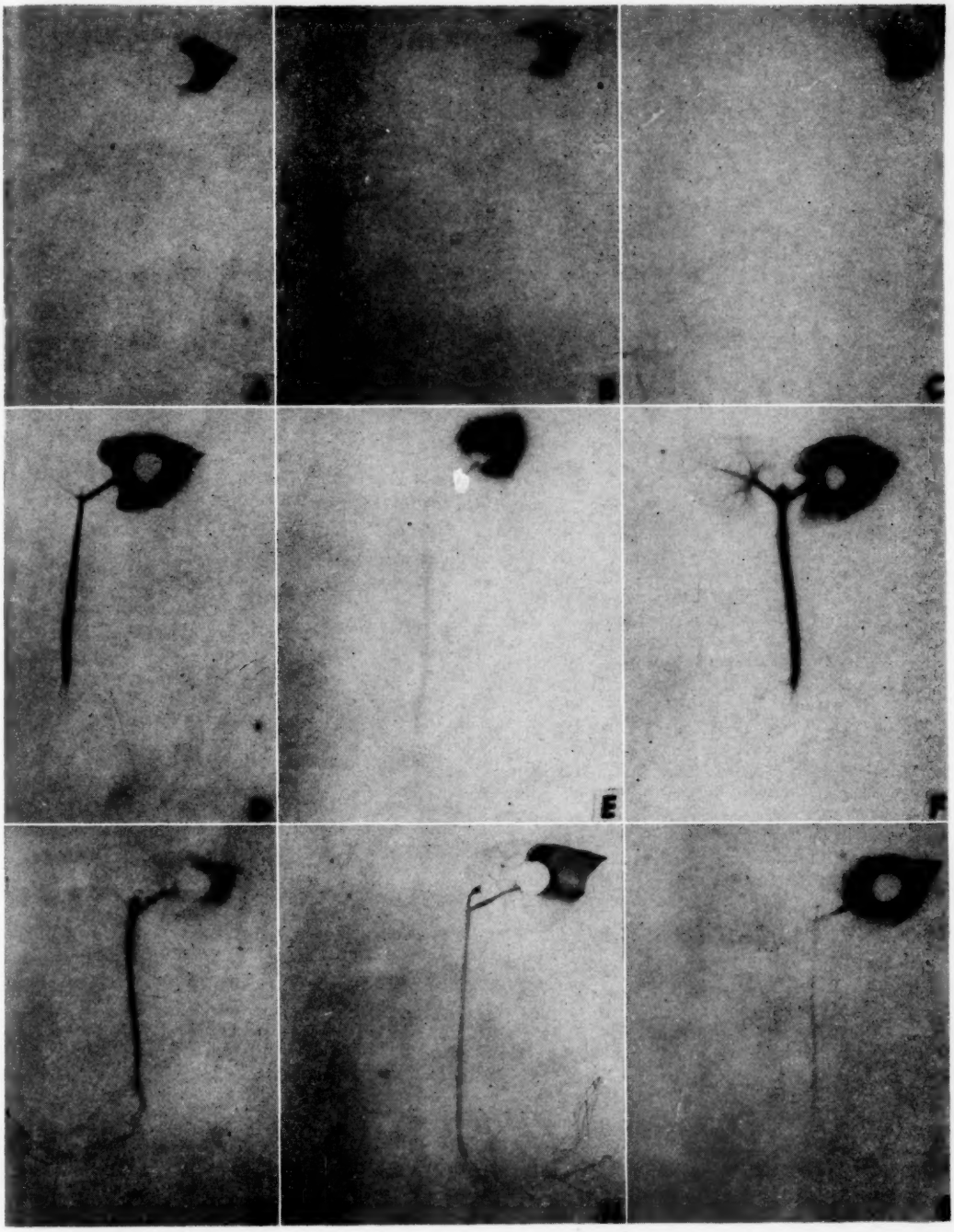

FIG. 4. Autoradiograms illustrating the distribution of Ca^{45} (A), Sr^{89} (B), $BaLa^{140}$ (C), Na^{22} (D), K^{42} (E), Rb^{86} (F), P^{32} (G), S^{35} (H), and Cl^{36} (I), 24 hours following application to one of the primary leaves of the bean plant. Since exposure times varied in the preparation of the autoradiograms, a strict quantitative comparison is not possible. (Disc removed from the leaf indicates site of isotope application.)

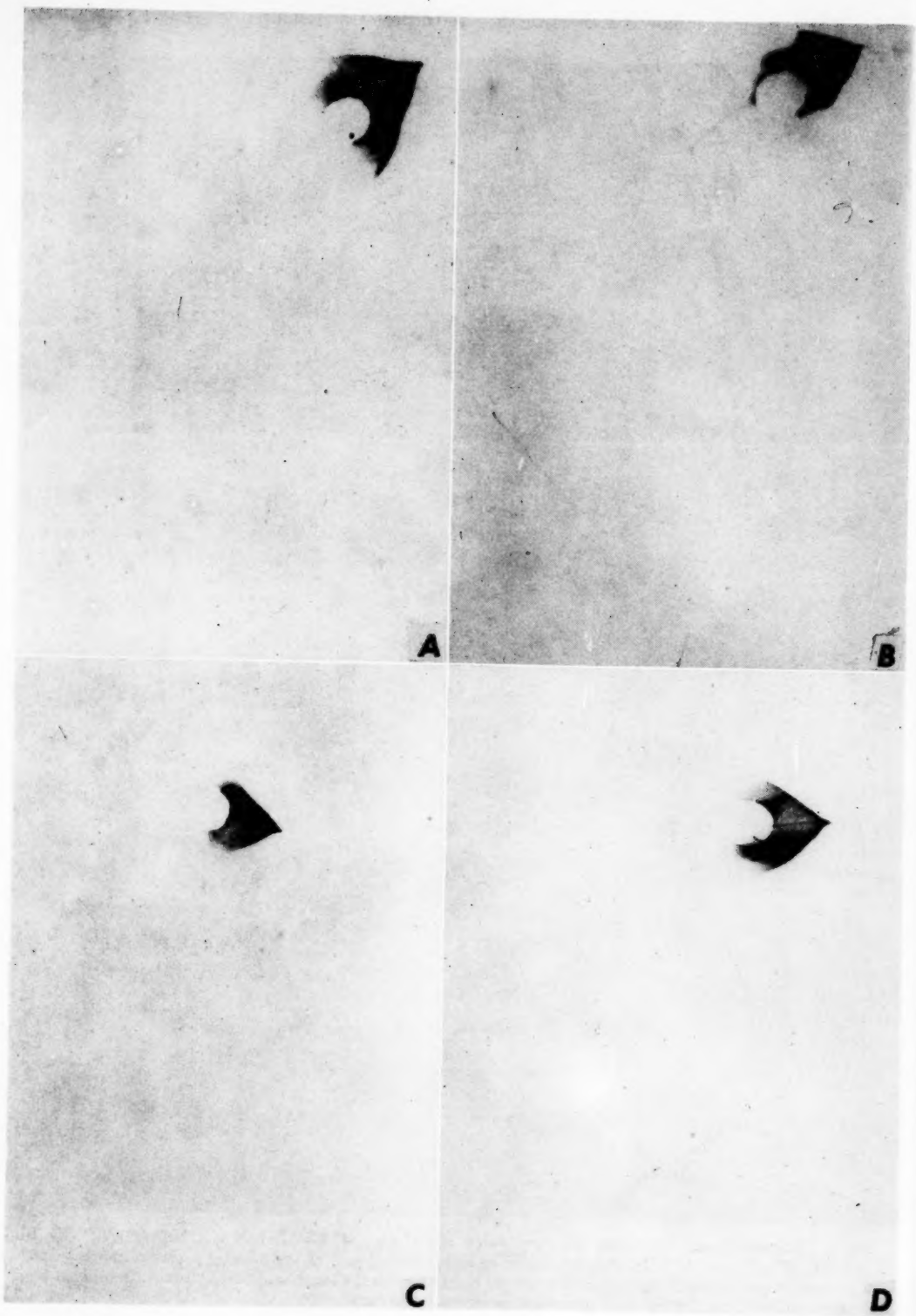
A
B
C
D

TABLE III

FOLIAR ABSORPTION * OF NUTRIENTS AS RELATED TO METHOD OF APPLICATION TO THE LEAF

NUTRIENT	ISOTOPE	LEAF DIPPED		DROPLET ON LEAF BLADE	
		STEM + NON-TREATED LEAF	ROOT	STEM + NON-TREATED LEAF	ROOT
Phosphorus	P^{32}	0.3	0.2	1.1	1.0
Sulfur	S^{35}	...	..	1.6	1.1
Chlorine	Cl^{38}	1.2	0.3	3.5	0.3
Sodium	Na^{22}	12.5	4.2	13.4	1.7
Potassium	K^{42}	11.2	1.2	10.2	0.8
Rubidium	Rb^{86}	15.2	5.8	16.1	6.5

* Expressed as % of applied nutrient recovered after 24 hrs in non-treated parts.

The absorption rates of calcium (Ca^{45}), strontium (Sr^{89}) and barium ($BaLa^{140}$) are closely parallel. Whereas transport from the site of application into adjacent leaf tissue is appreciable, export from the treated leaf is negligible. The almost complete absence of basipetal transport of Ca^{45} verifies previous reports by the authors (5, 6, 20) as well as others (2, 14). That strontium (Sr^{89}) and barium ($BaLa^{140}$) are also immobile has been determined by the absence of radioactivity in plant parts other than treated leaves, and is illustrated by the autoradiograms (figs 4-A, 4-B, 4-C). The immobility of calcium, strontium and barium is attributed to failure of phloem transport, and polar movement (20). When polarity is suspended by anesthetization with di-ethyl ether, basipetal transport (via the phloem?) occurs (5). Within the leaf, transport from the site of application occurs only toward the apex and periphery (figs 4-A, 4-B, 4-C). The possible role of transpiration in such movement has been suggested (22).

Zinc (Zn^{65}), copper (Cu^{64}), manganese (Mn^{52-54}), iron (Fe^{55-59}) and molybdenum (Mo^{99}) were intermediate in mobility between highly mobile phosphorus (P^{32}) and relatively immobile calcium (Ca^{45}), and with decreasing mobility in the approximate order listed. According to Wallihan and Heymann-Herschberg (18), zinc (Zn^{65}) was readily absorbed by and transported from the leaves of citrus. Romney and Toth (13) have reported that manganese (Mn^{54}) was absorbed through soybean leaves and translocated throughout the plants. Figures 2-D and 3 showing absorption and transport rates, and figure 5 the autoradiograms, suggest that with the exception of zinc (Zn^{65}) and possibly copper (Cu^{64}) there was little export from the treated bean leaf. Transport was largely confined to the adjacent leaf tissue and appeared to follow the transpiration stream. Appreciable basipetal transport did not appear evident up to 192 hours after treatment. Absorption values (figs 2-D and 3) for manganese (Mn^{52-54}), iron (Fe^{55-59}) and molybdenum (Mo^{99}) represent the amount of isotope recovered, almost exclusively, in the treated leaf minus the 1 cm disc (fig 1-B). Extreme variations in results precluded a listing of accurate absorption rates for Cu^{64}, however, the autoradiogram (fig 5-A) suggests that some export from the treated leaf occurred.

Several limitations are obvious in the materials and methods employed and in the results presented. 1) Processed formulations and irradiated units of the isotopes varied greatly in specific activities (table I). Thus, standardization of molar concentrations or ionic carriers of the solutions applied to the leaves was not feasible. However, this limitation was partially overcome by expressing absorption-transport values as percentages of the total isotope applied. 2) All absorption-transport rates were lower than the real values since the 1 cm leaf disc including and surrounding the site of application of each isotope was not included. Radioactivity recovered in other plant parts represented the absorbed and translocated element. 3) For absorption studies on sodium (Na^{22}), potassium (K^{42}), and rubidium (Rb^{86}), samples taken at intervals of less than 6 hours would have been desirable because of the rapid absorption and transport which occurred with these materials. 4) In the assay of plant samples for radioactivity, self-absorption of the weak beta emitters such as S^{35} and Ca^{45} by the dried plant tissue was not considered. This was determined to be of no great consequence on the nature of the results procured with the possible exception of S^{35}, since little or no Ca^{45} was exported from the treated leaf, and self-absorption by the leaf tissue itself was small. Self-absorption of S^{35} by the dried tissue undoubtedly resulted in values that were low, however, all radioactive values were relative and expressed as percentages of the total applied, and the results compared favorably with those of Biddulph, Cory and Biddulph (3) where greater precautions were taken to eliminate this variable.

It is apparent from these studies that all elements applied to leaves were absorbed and translocated but not at equal rates or in a comparable pattern. Any mass flow mechanism of transport was unlikely. The presumably free ions of sodium, potassium, rubidium and chlorine were rapidly absorbed by the bean leaf and a high percentage exported to other plant parts, especially into the stems.

While phosphorus, sulfur and possibly zinc were

FIG. 5. Autoradiograms showing the distribution of Cu^{64} (A), Zn^{65} (B), Fe^{55-59} (C), and Mn^{52-54} (D) 24 hours following application to one of the primary leaves of the bean plant. Since exposure times varied in the preparation of the autoradiograms, a strict quantitative comparison is not posible. (Disc removed from the leaf indicates site of isotope application.)

TABLE IV

CLASSIFICATION OF NUTRIENTS AS TO THEIR MOBILITY IN THE BEAN PLANT FOLLOWING FOLIAR APPLICATION *

MOBILE	PARTIALLY MOBILE	IMMOBILE
Rubidium	Zinc	Calcium
Sodium	Copper	Strontium
Potassium	Manganese	Barium
Phosphorus	Iron	.....
Chlorine	Molybdenum	.....
Sulfur	.....	.....

* Listed in order of decreasing mobility.

also highly mobile in the bean plant, either absorption or transport, or both, occurred at a slower rate than for sodium, potassium, rubidium or chlorine. Distribution of these nutrients within the plant appeared proportional to the metabolic activity of the various tissues. Calcium, strontium and barium were immobile, while copper, manganese, iron and molybdenum were readily absorbed by leaves but were only partially mobile—a high percentage of the absorbed isotope remained within the absorbing leaf, with the primary movement being toward the tip or periphery. Using as a criterion the percentage absorption and transport of the foliar applied isotopes from the site of application to the non-treated plant parts (figs 1-C and 1-D), and autoradiograms to depict distribution patterns within the plant at various intervals after treating, the nutrient elements studied are classified (table IV) in terms of mobility (percentage transport from the treated leaf into other plant parts).

SUMMARY

The absorption, transport and mobility of foliar applied radioactive isotopes of rubidium, sodium, potassium, phosphorus, chlorine, sulfur, zinc, copper, manganese, iron, molybdenum, calcium, strontium, and barium were determined with the bean as the experimental plant. Using as a criterion the percent of the foliar applied radioactive isotope recovered in non-treated plant parts, and autoradiography to portray gross distribution in the plant it was found that rubidium, sodium and potassium were the most readily absorbed and most highly mobile. Calcium, strontium and barium while absorbed by the leaf were not exported from the leaf and were considered immobile. Phosphorus, chlorine, sulfur, zinc, copper, manganese, iron, and molybdenum were intermediate with decreasing mobility in the order given.

Since the preparation of this manuscript, preliminary observations with magnesium (Mg^{28}) showed that it was immobile and its distribution following foliar application was the same as Ca^{45}, Sr^{89} or $BaLa^{140}$.

The assistance of Dr. C. C. Chen, in conducting some of the experiments is gratefully acknowledged.

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THE HILL REACTION OF RED KIDNEY BEAN CHLOROPLASTS[1,2,3]

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Red kidney beans produce large, uniform primary leaves in a short amount of time. Theoretically these should be excellent material for studying problems of leaf growth, etc. One aspect of the physiology of the leaf of interest to us was the ability of isolated chloroplasts to carry on parts of photosynthesis in vitro. We noticed that the Hill reaction activity (3, 4) of such chloroplasts was considerably lower than that of spinach chloroplasts regularly in use in this laboratory. The following report concerns our attempts to improve the Hill activity of chloroplasts from red kidney bean leaves by manipulating several variables of the leaf grinding procedure.

In the course of this study, evidence has been obtained which suggests that there are two pathways for dye reduction in the Hill reaction.

MATERIALS AND METHODS

Leaves of red kidney beans, black valentine beans, peas, oat, barley, cabbage, tomato, some ferns, sunflowers, cocklebur and vanilla were obtained from plants grown in the greenhouse. Spinach and parsley were obtained from the local supermarket. Seven to fifteen grams of leaf tissue were ground in 50 ml of buffer. When leaves were ground in the Omnimixer, it was run at 35% of line voltage for one minute. The homogenates were strained through cheesecloth and glass wool.

The basal medium for the grinding buffer consisted of: 0.30 M sucrose, 0.01 M KCl, and 0.05 M phosphate. The pH of the phosphate was varied as indicated in the text; and other additions to the medium were made at times.

All chloroplasts were centrifuged from the original homogenate at 1000 × g for 10 minutes, resuspended in 40 ml of buffer, and washed once at 1000 × g. No matter what the grinding medium, all chloroplasts were washed and resuspended in a medium containing 0.30 M sucrose, 0.01 M KCl, and 0.05 M phosphate pH 7.3.

For Hill reaction measurements, 0.072 micromoles of 2, 3, 6-trichloroindophenol dye (Eastman Kodak), 150 micromoles of TRIS (tris-(hydroxymethyl) aminomethene) buffer at pH 7.2, and an appropriate amount of chloroplast suspension in a total volume of 3.0 ml were incubated for 45 seconds in the light in a water bath at room temperature in front of a 100 Watt light bulb. The optical density at 620 millimicrons was determined before and after exposure to the light. Light intensity was considerably over saturation. The amount of chloroplasts was varied from 0.001 to 0.010 mg chlorophyll per ml of reaction mixture, in order to give a change of 0.090 to 0.120 in optical density in the 45-second period. The unit of activity from this measurement is defined as change in optical density at 620 $m\mu$ per minute. Final activities are all shown as units per mg chlorophyll. All determinations were done in duplicate or triplicate. Chlorophyll was determined by the method of Arnon (1).

RESULTS

The initial observation was that chloroplasts from spinach were regularly giving Hill reaction rates of 25 to 40 units per mg chlorophyll, while those from red kidney bean leaves showed rates of 3 to 9 units per mg chlorophyll. On the hypothesis that conditions obtaining in the grinding process were causing chloroplast inactivation, various "protective" compounds were added to the grinding medium. The first addition found effective in raising the activity of kidney bean chloroplasts was Versene (ethylenediamine tetraacetic acid). In a typical experiment, activity of chloroplasts from a medium without Versene was 4.2 per mg chlorophyll; when Versene was used at 0.01 M in homogenizing, activity of the resulting chloroplasts was 14.2 units per mg chlorophyll. Versene (0.001 M) had no effect.

The pH of the grinding medium is of considerable

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TABLE I

EFFECT OF pH OF HOMOGENIZING BUFFER AND ADDITION OF PROTECTIVE AGENTS ON HILL ACTIVITY OF KIDNEY BEAN CHLOROPLASTS

pH OF GRINDING MEDIUM	ADDITION TO GRINDING MEDIUM	HILL REACTION, UNITS/MG CHLOROPHYLL
7.3	.....	7.4
7.3	Versene	18.3
8.5	.....	14.1
8.5	Versene	18.4
8.5	Versene, PVP	20.8

Versene added was 0.01 M; PVP, 3 %.

importance, as can be seen in figure 1. The pH optimum for the grinding medium appears to be between 8.0 and 8.5. This is higher than the pH of the buffers previously used for grinding leaves in this laboratory, which were about 7.0 to 7.3.

It was necessary to see if a high pH entirely eliminated the need for Versene. That this is not true is seen in table I. In this experiment a higher pH raised the rate from 7.4 to 14.1, and Versene increased it to 18.4.

Another protective agent that has come into use recently is polyvinylpyrrolidone (PVP) (2). Our results with this material in the grinding medium were variable, from no extra effect to slight protection (table I). Since it never had a deleterious effect and often gave slight extra protection it was occasionally incorporated into the final proective medium at 3 %. The other components of the final protective medium are 0.01 M Versene, 0.05 M phosphate buffer pH 8.5, 0.01 M KCl and 0.30 M sucrose.

Other possible protective agents that were tried, but which had no added effect, were: cysteine, ascorbic acid, citric acid, serum albumin, and sodium pyrophosphate. Carbowax (7) was not tried, because it had been shown to cause precipitation of proteins otherwise soluble.

All of the experiments so far reported represent red kidney bean leaves ground in the Omnimixer, at low speed. During the period from May to December of 1956, in experiments repeated many times over, it was found that grinding leaves in a mortar and pestle resulted in damage to the Hill reaction of the chloroplasts. Since December of 1956, it has not been possible to repeat this observation; and as of now chloroplasts are just as active whether ground in a blendor or mortar and pestle. It has not been possible to discover the reason for this difference, although it seems to be associated with a new lot of seeds purchased at about that time. Since the phenomenon is not now repeatable, data for the chloroplasts damaged by mortar and pestle grinding will not be reported; however, in all respects they seemed to resemble chloroplasts damaged by grinding in the absence of Versene, at pH 7.2.

These findings left us with two kinds of chloroplasts: a) of low activity, due to grinding at pH 7.3 and without Versene, and b) highly active, as a result of grinding at pH 8.5, with Versene present, in an Omnimixer or blendor. A search was made for superficial differences between these preparations which might explain the difference in activity, but none was found.

Specifically, the difference in activity between the two types of chloroplasts is not an artifact due to the existence of an unusual lag period in one case and not in the others. The time course of the reactions, studied at 10-second intervals, were found to be essentially identical.

The difference between the two types is not limited to whole chloroplasts which were used for most of the experiments. The same relationship was found if the

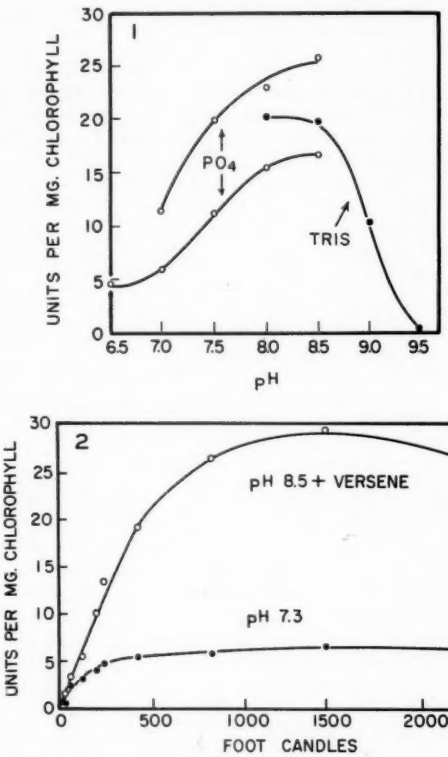

FIG. 1 (*top*). Effect of pH of the homogenizing buffer on Hill activity of isolated chloroplasts. The medium contained sucrose, KCl, and 0.05 M buffer salts of the kind and pH indicated. The three curves are from three different experiments.

FIG. 2 (*bottom*). Hill reactions as a function of light intensity. The light intensity was varied by altering the voltage to the light source (a 100-watt tungsten lamp) with a Variac; and foot candles at each voltage were determined with a light meter. Reaction mixture for pH 7.3 chloroplasts contained 0.0031 mg clorophyll per ml, with pH 8.5 + Versene chloroplasts contained 0.0039 mg chlorophyll per ml.

TABLE II

EFFECT OF GRINDING PROCEDURES ON DIFFERENT CHLOROPLAST FRACTIONS

GRINDING PROCEDURE	HILL REACTIONS, UNITS/MG CHLOROPHYLL		
	WHOLE HOMOGENATE	CHLOROPLASTS	GRANA
pH 7.3	13	9	8
pH 8.5	47	33	45

Chloroplasts were centifuged out of suspension at 1000 × g for 10 min. Grana were collected by centrifuging the supernatant at 20,000 × g for 15 min.

whole, uncentrifuged homogenate was used as the enzyme, or if "grana" were collected from the homogenate, instead of chloroplasts (table II).

The lower activity of the poorer preparation is not attributable to a more rapid re-oxidation of the reduced dye, but actually represents a slower rate of reduction. The same order of activities was found if the reactions were performed anaerobically. In addition, no visible sign of significantly rapid dark re-oxidation of the dye was ever observed, in air.

The low activity of the chloroplasts prepared at pH 7.3 was not raised at all by pre-incubation with the dye, or by raising the dye concentration to double its usual level. It therefore seems unlikely that the slower reaction is due to a slower rate of penetration of the dye into the chloroplast. This conclusion is also supported by the failure of sonic oscillation to cause any increase in the rates of the poorly active chloroplasts (fig 3).

As shown in table III, buffer at pH 7.3 in the absence of Versene does not influence chloroplasts adversely, once they are removed from the homogenate. Alternatively, once a leaf has been ground at pH 7.3 in the absence of Versene, adding Versene and raising the pH does not restore high Hill activity to the chloroplasts. At the time when chloroplasts were damaged by grinding the leaf in a mortar and pestle, no damage could be done to isolated chloroplasts or even to a freshly ground homogenate, by re-grinding in a glass homogenizer. It would appear, therefore, that the activity of the chloroplasts is established at the time of the initial grinding of the leaf and reversing the treatments after that point cannot change the activity of the plastids.

TABLE III

INABILITY OF GRINDING TREATMENTS TO AFFECT ISOLATED KIDNEY BEAN CHLOROPLASTS

HOMOGENATE	HOMOGENATE* TREATED	CHLOROPLASTS TREATED	HILL REACTION, UNITS/MG CHLOROPHYLL
pH 7.3	.....	.....	15
	pH 8.5 + Versene**	.....	14
	.....	pH 8.5 + Versene	10
pH 8.5 + Versene	.....	.....	33
		pH 7.3	30

* The whole homogenate resulting from the first leaf grinding was re-treated as shown, before straining through cheesecloth and glass wool, so that all of the original components were still present. Chloroplasts were re-treated after their first centrifugation out of the homogenate, but before they were washed. After treatment they were centrifuged, and all preparations finally re-suspended in the usual phosphate-sucrose-KCl buffer at pH 7.3.

** The pH of this homogenate was raised to 8.5 by adding NaOH, and 0.01 M Versene was added.

A brief survey was made of a number of other plants to see whether their chloroplasts reacted in the same way to the grinding medium. The results with fourteen other species are shown in table IV.

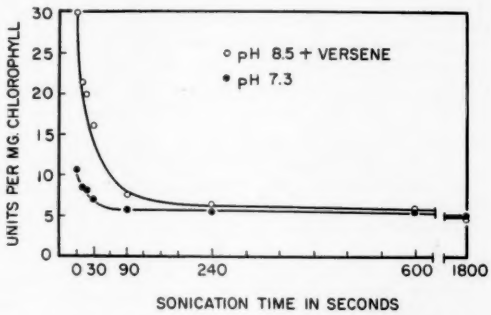

FIG. 3. Effect of sonic oscillations on Hill reactions of three types of chloroplasts. Sonic oscillation was performed in a Raytheon magnetostrictive 20-Kc oscillator, at 1.0 to 1.1 amperes. The chloroplasts were cooled to 0° C during oscillation. The chlorophyll concentrations during treatment were between 0.056 and 0.086 mg/ml. Separate 10-ml aliquots of chloroplasts were vibrated for the times shown, then Hill reaction rates determined as soon as possible afterwards. Chlorophyll concentrations in the final reaction mixtures varied from 0.0017 to 0.0056 mg/ml, depending on the chloroplast activity.

Chloroplasts from vanilla and the three species of ferns showed low activities, and no change in the grinding procedure improved matters. With a number of plants (kale, parsley, barley, oats, pea), activity was high under all conditions. The protective medium gave marked improvement in activity of chloroplasts from black valentine bean, tomato, sunflower, cocklebur and cabbage.

The Hill reactions of bean chloroplasts were measured at a series of different light intensities, in order to gain some idea of the site of damage caused by improper grinding medium. The results of one experiment are shown in figure 2; another experiment confirmed the essential points of the curves. It can be seen that the "damage" to the Hill reaction amounts to 80 % at the saturating light intensities, but only 30 to 40 % in limiting light (at 59 ft-c). Alternatively, on plotting these curves as 1/rate vs 1/light intensity, straight lines are found with parallel slopes.

TABLE IV

RESPONSE OF DIFFERENT PLANTS TO GRINDING MEDIUM

PLANT USED	HILL REACTION RATES UNITS/MG CHLOROPHYLL	
	pH 7.3 *	pH 8.5 *
Black Valentine Bean	12.0	25.1
Sunflower	9.3	34.0
Cabbage var. Copenhagen Market	11.9	22.4
Tomato var. Rutgers	6.3	23.4
Cocklebur (flowering)	9.6	21.7
Adiantum pedatum	3.9	5.2
Pellea sp.	3.0	3.0
Polypodium virginianum	2.0	3.8
Vanilla	4.7	3.2
Kale	30.7	33.0
Oats	30.2	24.1
Pea var. Tall Telephone	25.5	32.0
Parsley	18.5	21.3
Barley	17.5	21.3
Spinach	32.2	24.1

* pH 7.3 indicates the basal grinding medium, containing 0.30 M sucrose, 0.01 M KCl, and 0.05 M phosphate buffer pH 7.3. pH 8.5 indicates the same medium plus the addition of 0.01 M Versene and 3 % PVP, the whole raised to pH 8.5.

These data indicate quite clearly that the damage by improper grinding procedures affects primarily a dark step in the Hill reaction.

Further evidence for the nature of the damage to bean chloroplasts comes from subjecting the two kinds of preparations to sonic oscillations for varying periods of time. The resulting activities are plotted in figure 3. It can be seen, first of all, that the highly active chloroplasts are very sensitive to sonic oscillations, losing 50 % of the activity that they are going to lose in about 30 seconds. (The speed of inactivation here is lowered by increasing the concentration of chloroplasts during sonication.) Secondly, the activity does not drop to 0, but rather to a specific activity of about 5, in 4 minutes. Further vibration up to 30 minutes has no further effect. Thirdly, and of great interest, is the fact that the less active chloroplasts are much less sensitive to sonic oscillation. Indeed, they drop from wherever their activity is, to practically the identical level as the sonicated active chloroplasts. This drop is extremely rapid, and then no further change occurs even after 30 minutes of further sonication.

An attempt was made to see if the damage caused by sonic oscillation could be related entirely to breaking the chloroplasts down below the "minimum particle size" (8). Control, and sonicated chloroplasts were subjected to centrifugal fractionation immediately after sonication. Fractions were collected at $2000 \times g$ for 10 minutes, $7000 \times g$ for 10 minutes, $20{,}000 \times g$ for 30 minutes. The supernatant from this last centrifugation was also assayed. Semiquantitative procedures were used in order to get some idea of total recovery of activity. As seen in table V, total recovery of the chlorophyll was possible, but not of Hill reaction activity. It is likely that most of the unrecovered activity was lost from the smaller fractions, since these were centrifuged at stronger forces, and their activities had to be measured at a longer time after sonication.

If damage was due entirely to gross disruption of chloroplast particles, it could be expected that any chloroplasts not broken up would have the same activity as the control chloroplast. This is not the case, as can be seen in Table V; even the largest particles from a sonicated preparation had lower activity than the unsonicated chloroplasts. Microscopic observation showed that this large particle fraction contained visible whole chloroplasts. Although it is not entirely impossible that these chloroplasts lost activity during centrifuging, the chances of this are minimized since they required the shortest period of centrifugation, at the lowest speeds.

DISCUSSION

The improved medium for the isolation of bean chloroplasts has resulted in raising the Hill reaction rates from the low values of 3 to 6 units/mg chlorophyll first observed, to higher values between 20 and

TABLE V

CENTRIFUGAL FRACTIONATION OF SONICATED KIDNEY BEAN CHLOROPLASTS

SONICATION TIME, SEC	FRACTION	CHLOROPHYLL MG/ML	HILL REACTION	
			UNITS/ML	UNITS/MG CHLOROPHYLL
0	Original particles	0.140	2.98	21.3
	$2{,}000 \times g$	0.136	2.42	17.8
	$7{,}000 \times g$	0.003	0.08	22.4
	$20{,}000 \times g$	0.001	0.01	11.1
	$20{,}000 \times g$ supernatant	0.000	0.00	...
	Recovery	0.140	2.51	
60	Original particles	0.140	2.17	15.5
	$2{,}000 \times g$	0.088	1.09	12.4
	$7{,}000 \times g$	0.013	0.20	15.0
	$20{,}000 \times g$	0.019	0.19	9.9
	$20{,}000 \times g$ supernatant	0.020	0.12	6.0
	Recovery	0.140	1.60	
180	Original particles	0.140	1.20	8.6
	$2{,}000 \times g$	0.014	0.13	7.5
	$7{,}000 \times g$	0.011	0.10	8.3
	$20{,}000 \times g$	0.034	0.25	7.4
	$20{,}000 \times g$ supernatant	0.070	0.37	5.3
	Recovery	0.129	0.85	

Centrifugation at $2{,}000 \times g$ was for 10 min; at $20{,}000 \times g$ was for 30 min. The supernatant from the first centrifugation was used for the next, and so on. Pellets were resuspended in known volumes of buffer. The leaves had been ground in the Omnimixer at pH 8.5, and the chloroplasts were washed once before sonication.

30. This compares favorably with the rates seen with spinach chloroplasts, which under the conditions used have rates of 25 to 40. A rate of 30 Beckman units/mg chlorophyll × minute corresponds to the reduction of 304 micromoles of dye/mg chlorophyll × hr,[4] or the liberation of 3,400 microliters of oxygen/mg chlorophyll × hr. These values are somewhat higher than those usually reported (6); it is probably due to the fact that the reaction was measured for a period of only 45 seconds, at room temperature (which varied from 23 to 30° C). Under these conditions it is possible to measure rapid initial rates, without complications due to chloroplast inactivation during the reaction. Also the low concentrations of the oxidant avoids secondary poisoning or stimulating effects which are found at the high concentrations required for manometric measurements (5).

It should be emphasized that the conditions that were manipulated in this study were only those that occurred during the original homogenizing procedure. Addition of protective reagents in the washes did not affect the final rates of Hill reactions observed. Once the activity of the chloroplasts is established by the initial grinding procedure, the same or alternative treatments do not seem to be able to change the level of dye reduction.

The function of Versene as a protective agent is most probably related to its chelating ability. It is therefore possible to infer, tentatively, that there are metal ions or metal containing enzymes in the homogenate that cause damage to the chloroplast. The effect of raising the pH of the buffer is not so easy to explain. Bean leaves ground in distilled water have a pH of 6.0. With 0.05 M phosphate at pH 7.3 used for grinding, the homogenate pH is 7.0, if pH 8.5 phosphate is used, the homogenate pH is 7.5. There are many possibilities in regard to the condition of compounds in solution in the homogenate, and activity of potentially dangerous enzymes, which might be invoked to explain the protection due to a higher pH.

Whether these two agents protect chloroplasts or not would depend on the contents of the leaf cells liberated during grinding, and would therefore vary with the particular leaf. It is not surprising, then, that of plants with low Hill activity, five (bean, tomato, cabbage, sunflower and cocklebur) respond to these protective agents, where the three ferns do not.

The nature of the damage due to an improper grinding medium is entirely unknown. The data presented here do not eliminate the possibility that a photo-oxidation of the dye has been induced, as the cause of the net decrease in dye reduction. While the rate vs light intensity curves make it appear that a dark reaction of photolysis is affected rather than the initial light-dependent step, still about the same result might be predicted from a light-dependent photo-oxidation that is more effective at high than at low light intensities. Since performing the Hill reactions under nitrogen does not relieve the inhibition on the part of "damaged" chloroplasts, any such photo-oxidation would have to use the oxidizing part of the Hill reaction, rather than atmospheric oxygen, as electron acceptor. The only evidence against the photo-oxidation concept is the fact that the rate of reduction by damaged chloroplasts is much lower than that of active chloroplasts even during the first ten seconds of the reaction, when the ratio of oxidized to reduced forms of the dye drops from infinity to about 10. This means that any hypothetical photo-oxidation would have to proceed just as rapidly at 1×10^{-6} M dye and below as the photo-reduction does between 2×10^{-5} and 1×10^{-5} M.

The effect of sonic oscillation on these chloroplasts is entirely unlike that reported by Thomas, Blaauw, and Duysens (8). In the report from the Dutch laboratory, chloroplast activity was quite unaffected by sonic oscillation until a minimal, sub-microscopic size was reached; then further reduction in size resulted in complete loss of activity. Unfortunately, the two sets of experiments are really not at all comparable. Our temperature during oscillation was 0° C, theirs was 12° C. Oscillation here was in air; Thomas et al (8) found complete loss of activity due to aerobic oscillation in 60 seconds, and so performed their oscillation anerobically. We measured chloroplast disruption as a function of the length of sonic oscillation; the report of the Dutch group does not state how varying degrees of disruption were obtained, but these varying degrees were apparently obtained in different experiments with different leaves. They measured the size of the residual particles in the electron microscope in each case, which we did not do. We started out with whole chloroplasts, washed once; they started out with grana, evidently unwashed. Hill reaction measurements here were done with the indophenol dye at a concentration of 2×10^{-5} M over a 45-second period; the Dutch group used benzoquinone at about 2.5×10^{-3} M and made manometric measurements presumably over a 15- to 30-minute period.

It is clear, therefore, that the conflicting results obtained by no means challenge the concept of a "minimum particle size," derived from their sonic oscillation experiments. It does seem, however, that this particular concept cannot explain the rapid damage caused to highly active red kidney bean chloroplasts by sonic oscillation. The suspensions of chloroplasts were becoming visibly more clear between 2 minutes and 30 minutes; nevertheless, the Hill activity was barely disturbed during this time. In particular, even the whole chloroplasts left after a brief sonication period had lower activity as a result of the sonic oscillation. It is likely that some sort of damage to the internal structure occurred in these chloroplasts; and the internal damage caused the lowering of dye reducing ability.

The most unusual aspect of sonic oscillation is that it reduces the activity of chloroplasts to a lower level, which is then resistant to further oscillation.

[4] Using a millimole extinction coefficient of 17.7 for indophenol dye at 620 mμ.

In addition, damage by the harmful homogenizing treatments and damage by sonication are not additive; chloroplasts prepared at pH 7.3 are inactivated further only a very small amount by sonication. This makes it appear that the harmful homogenizing treatments, and sonic oscillation, attack at primarily the same site.

These facts lead us to suggest that there may be two pathways for dye reduction in the Hill reaction of red kidney bean chloroplasts. One of these pathways is susceptible to damage by sonic oscillation, or by a pH below 8 during homogenization; the second pathway is resistant to these treatments. The very high rates of dye reduction which can be observed under optimal conditions represent the sum of reduction along both pathways.

A similar conclusion as to the complex nature of the reducing pathway in the Hill reaction has been arrived at independently by T. Punnett on the basis of the pH optimum of different kinds of chloroplasts.

Summary

The Hill reaction rates of red kidney bean chloroplasts prepared by usual procedures were found to be low compared to those of spinach. Active chloroplasts could be prepared only if Versene was added to the homogenizing buffer. Raising the pH of the grinding buffer to 8 or over could substitute in part for the presence of Versene.

Once the leaves were ground, no further changes in chloroplast activity could be induced by reciprocation of the above treatment. Chloroplasts with low activity have apparently been damaged in a dark step, not in the light dependent part of the Hill reaction.

Sonic oscillation has only a slight effect on chloroplasts already damaged by a harmful homogenizing medium. On the other hand, it causes a rapid loss of 80% of the activity of highly active chloroplasts. After brief sonication both types of chloroplasts are reduced to the same activity which is then resistant to extended additional sonication.

Consideration of these phenomena leads to a suggestion that there may be two pathways for dye reduction in the Hill reaction of red kidney bean chloroplasts, one sensitive, and one resistant to these inactivating conditions.

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EFFECT OF GIBBERELLIN ON GROWTH, FLOWERING AND FRUITING OF THE EARLYPAK TOMATO, LYCOPERSICUM ESCULENTUM[1,2]

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Gibberellin applied to tomato plants has been shown to induce marked stem elongation (4), to increase fresh weight (4, 15), to accelerate flowering and produce greater numbers of flowers per plant (17), and to increase fruit set (15, 17). This paper describes more definitively, the influence of gibberellins on growth, flowering, and fruit set of the Earlypak tomato.

General Procedure

Tomato seed (var. Earlypak, Ferry-Morse Seed Company, Salinas, California) was germinated in flats of vermiculite. Before the first true leaf expanded, uniform plants were transferred to flats or cans and grown in a greenhouse held at 18° to 21° C or in an outdoor lath house. Plants were grown to fruit maturity in soil benches or in large cans of soil.

A mixture of gibberellic acid ($[\alpha]D+92°$) and gibberellin A (Ga) ($[\alpha]D+36°$) (9) was dissolved in 0.5% ethyl alcohol or in water. In the experiment dealing with growth response 0.05 ml of the mixture of gibberellins was applied with a micropipette to the youngest developing leaves (when about 2 cm in length) and to the adjacent vegetative apex. Similar applications were made during later growth. An atomizer was used to spray flowers and developing fruits.

In the experiments concerned with stem heights,

[1] Received revised manuscript April 30, 1957.
[2] This paper is based on Project 1175 D.

measurements were taken at the appearance of the first flower clusters, and the distance between the cotyledonary node and the node below the first flower cluster is reported.

Experimental

Stem Elongation: The influence of repeated applications of Ga at specific intervals during growth was investigated. Using tomato plants grown in cans of soil, 25 μgm of Ga was applied to developing leaves at each of certain nodes as they appeared (table I). The final application coincided with anthesis of the first flower cluster. In plants treated at the first node increases in stem elongation were observed within a week. However, treatment of the succeeding nodes produced no observable stem height increases, and by anthesis of the first flower cluster no significant differences in stem elongation had resulted.

Table I

Stem Elongation and Flowering of Earlypak Tomato Plants as Influenced by Single or Repeated Applications of Gibberellin at the Indicated Nodes

Nodes treated	Times treated	Total gibberellin applied	Total ht	No. of nodes preceding 1st flower cluster	Time from seeding to flowering
no.	*no.*	*μgm/plant*	*cm*		*days*
...	0	0	45	7	53
1	1	25	45	8	49
1,4	2	50	48	9	48
1, 4, 8	3	75	46	8	48
1, 4, 8, 12	4	100	51	8	48
L.S.D. at 1 %			N.S.*	N.S.*	0.7

* N.S. = Not significant.

The effect of higher concentrations and repeated dosages was determined by treating tomato plants twice weekly with 0, 25, 50, 100 or 200 μgm per plant until the first flower cluster appeared. Repeated treatment with 25 μgm and 50 μgm per plant did not significantly affect stem elongation; with 100 and 200 μgm per plant, however, slight and marked differences, respectively, were obtained (table II).

The surprising lack of response by anthesis to single or repeated applications of 25 and 50 μgm per plant (tables I and II) and the longer stems resulting from repeated applications of 100 and 200 μgm per plant of Ga indicated a further study of the growth responses of young Earlypak tomato plants. Single applications of 2.5, 25, and 50 μgm of Ga per plant to the first expanding leaves stimulated stem elongation markedly. In contrast to elongation (measured at anthesis of the first flower cluster) of plants treated repeatedly until anthesis (tables I and II), significant effects resulted from single applications with 2.5 or 25 μgm per plant when plants were measured during early growth (table III). Stem

Table II

Effect of Gibberellin * on Elongation and Flowering of Earlypak Tomato Plants

Gibberellin, μgm/plant	Ht, cm	No. of nodes preceding 1st flower cluster	Days to flowering
0	47.0	8	58
25	49.0	8	54
50	54.6	8	53
100	58.7	8	52
200	70.5	8	59
L.S.D. at 1 %	10.4	N.S.**	3.7

* Application made twice weekly from the appearance of the first expanding true leaves until the appearance of the first flower cluster.
** N.S. = Not significant.

height increased no more with 50 μgm per plant, however, than with 25 μgm per plant.

In a more detailed study, concentrations of 0, 15, 150, 300, and 450 μgm per plant were applied to the first exapnded leaf. Six days after treatment plants receiving 150 to 450 μgm, as compared with untreated plants, elongated significantly. The greatest stem height increase resulted from applications of 450 μgm per plant (fig 1).

Fresh and Dry Weights: Reports regarding the influence of gibberellins on fresh and dry weight are inconsistent (1, 4). Since such increases in response to an applied chemical would be extremely significant, fresh and dry weights were determined. Ga at 50 μgm per plant was applied to the first expanded leaves of Earlypak tomato plants. Ten days later, these plants, together with some which received no Ga, were cut at the cotyledonary node and weighed. Fresh weight was 61 % more in the treated plants.

In another study the shoots of plants given 2.5, 25 or 50 μgm of Ga were cut and weighed 17 days after treatment. Fresh-weight (grams) increases over the control were obtained with all treatments; however, there was no significant difference between treatments with 2.5 and 25 μgm of Ga (table III). The shoots

Table III

The Influence of a Single Application of Gibberellin Applied to the First Expanding True Leaves on Stem Heights, Fresh Weights, and Dry Weights of Earlypak Tomato Plants

Gibberellin	Days after treatment			Fresh wt per plant	Increase in fresh wt over control	Increase in dry wt over control
	9	13	15			
μgm/plant	*Height, cm*			*gm*	*%*	*%*
0 (control)	2.5	4.8	7.5	3.8	...	...
2.5	4.9	8.0	11.3	4.8	26.1	18.5
25	8.3	12.0	15.4	4.5	18.1	14.8
50	9.2	13.3	16.8	5.3	40.0	40.7
L.S.D. at 1 %	2.7	5.9	5.2			

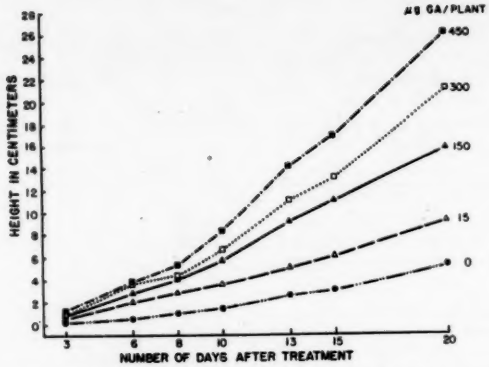

FIG. 1. The effects of 0, 15, 150, 300 and 450 μgm of gibberellin per plant applied to the vegetative apex on stem elongation of Earlypak tomato plants.

were placed in a 70° F forced-draft oven and dried for 24 hours. The percentage of dry matter of treated plants did not vary significantly from that of the controls. However, increases in total dry matter (% dry wt × fresh wt per plant) were obtained, especially with the 50 μgm application. The differences in fresh and dry weights between treatments at 2.5 and 25 μgm were not significant.

MORPHOLOGIC EFFECTS: Repeated foliar applications at concentrations above 25 μgm per plant frequently resulted in abnormal extension of the leaf rachis and leaflet petioles and in chlorosis of the leaves (1). In addition, the normally incised tomato leaves frequently developed entire margins (fig 2). Leaflets frequently rolled inward along the central vein after spraying at high concentrations. Axillary growth (tillers) decreased as concentration increased from 50 to 200 μgm per plant. At higher concentrations of Ga, spindly, weak plants developed. When

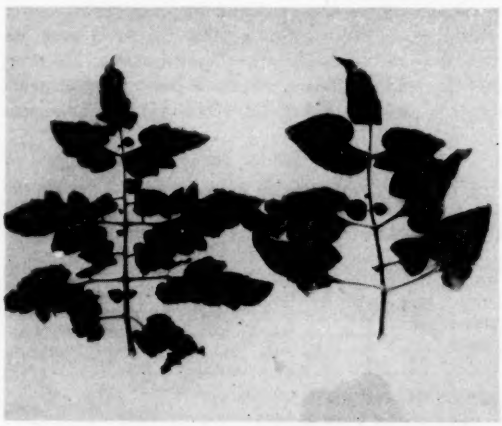

FIG. 2. Morphologic effects of gibberellin at concentrations above 500 μgm/ml. Notice the continuous margins, extension of leaflet petioles, and fewer leaflets per blade of treated leaf on the right.

early developing flowers of the first cluster were sprayed before anthesis with concentrations above 50 μgm, pedicels, sepals, petals, and pistils became enlarged. Fruits from such flowers usually developed abnormally.

FLOWERING: Wittwer and Bukovac (17) showed that while flowering in certain determinate tomato varieties may be hastened by early application of gibberellins, flowering of indeterminate varieties is relatively unaffected. Earlypak, like Pearson from which it is derived, is a determinate variety.

Treatment of plants at specific nodes with 25 μgm (table I) or twice weekly with 25, 50, or 100 μgm hastened flowering significantly without affecting the number of nodes below the first flower cluster (table II). No significant differences in flower numbers in the first two clusters resulted from applications of 0.05, 0.5, 2.5, or 25 μgm/ml of Ga made at the appearance of the first expanded leaf. Applied to the vegetative apex of tomato plants at first an-

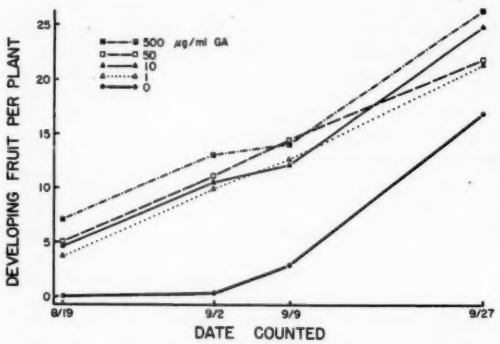

FIG. 3. The pattern of fruit set from the appearance of early developing fruit to the appearance of the first ripe fruit as affected by flower sprays of 0, 1, 10, 50 and 500 μgm/ml gibberellin.

thesis, similar treatments failed to affect subsequent flowering or fruit numbers.

FRUIT SET: Commencing August 4, 1956, the second and succeeding four flower clusters of tomato plants were sprayed twice weekly (for a period of 8 weeks) with Ga at concentrations of 0, 1, 10, 50, and 500 μgm/ml. Figure 3 shows the total number of developing fruits counted (per plant) preceding the appearance of the first ripe fruits. Figure 4 indicates the number of ripe normal and parthenocarpic fruit per plant harvested on specific dates. The fruit set of all sprayed flower clusters was rapid and almost complete, but in untreated plants fruiting was largely delayed until the appearance of the 5th or 6th cluster. Total fruit set of unsprayed plants never reached that of plants treated with gibberellin (fig 3).

Statistical analysis revealed that sprays of 1 μgm/ml were as effective as 500 μgm/ml in setting Earlypak tomatoes. The decrease in production of ripe normal fruit (fig 4) resulted from the frequency of harvesting (10/11 and again on 10/14) rather than

from any apparent differences in the pattern of fruit set. The average numbers of ripe normal fruit harvested per plant are shown in table IV. Ga sprays frequently induced parthenocarpy (fig 4 and table IV) with associated underdevelopment and poor ripening characteristics. However, when seeds were present in fruits from sprayed flowers, fruits generally developed normally.

In one experiment, spraying fruits twice weekly did not appear to affect fruit size, although the vegetative portions (calyx and pedicel) were characteristically enlarged. The restricted plant-growing area provided in 5-gallon cans of soil made the recording of fruit weights valueless.

Discussion and Conclusions

Rapid stem elongation is seen in many plants after treatment with extremely minute amounts of the gibberellins (1, 2, 3, 4, 5, 6, 9, 13, 14, 15, 18). With Earlypak tomato, although minor differences are apparent soon after treatment, pronounced stem height increases are obtained primarily with very high concentrations of Ga within 10 days after application. Stem elongation is marked when the first expanded leaf is treated during early growth (4 to 6 leaves), although by anthesis neither single nor repeated applications of up to 50 μgm of Ga produced significant differences in elongation. By anthesis only with repeated applications at concentrations above 50 μgm did stem elongation differ significantly from that of untreated plants. The results demonstrate the decreasing responsiveness to Ga as plants develop. A

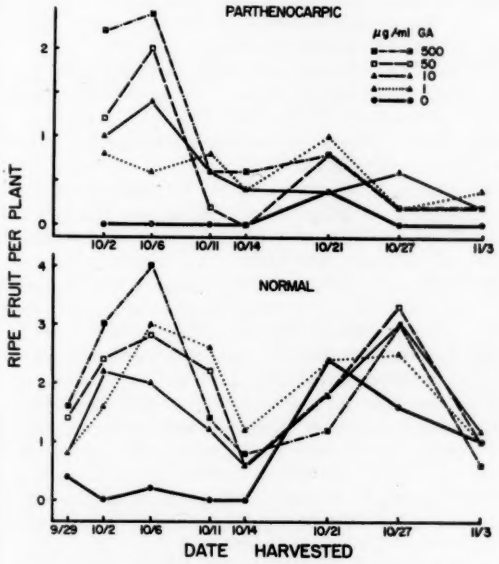

Fig. 4. The influence of flower sprays containing 1, 10, 50 and 500 μgm/ml of gibberellin applied twice weekly on the numbers of ripe normal and parthenocarpic fruit harvested at the indicated dates.

Table IV

Average Number of Normal and Parthenocarpic Ripe Earlypak Tomatoes Harvested from Sept. 20 to Nov. 3, 1956 Following Twice-Weekly Sprays of Gibberellin

Gibberellin, μgm/ml	No. of fruit/plant	
	Normal	Parthenocarpic
0	3.4	0.4
1	13.6	4.8
10	11.8	4.6
50	12.4	4.4
500	15.8	7.4
L.S.D. at 1%	5.6	1.4

possible explanation for the disparity between these results and those reported by Bukovac and Wittwer (4) is that they collected samples earlier in growth when differences in stem elongation were also apparent in the present studies. These differences, however, were negated by the time anthesis of the first flower cluster had occurred, possibly because of the decreasing receptiveness of older plants to Ga. In addition, important varietal differences occur. In concurrent studies plants of the Potentate variety were taller by anthesis when treated during early growth with 50 μgm per plant of gibberellic acid.

Increases in fresh and dry weights of plants grown with a continuous supply of gibberellin in water culture were reported by Brian et al (1) and Marth and coworkers (13). Bukovac and Wittwer reported a 50% increase in fresh and dry weight of celery (4). However, working with several tomato varieties, they did not obtain dry-weight increases with as much as 20 μgm per plant. In the present experiments Ga stimulated consistent stem elongation, leaf enlargement (rachis and leaflet petioles), and fresh-weight increases. Dry matter content (% dry wt × fresh wt per plant) was increased, especially when very high concentrations were used. The later harvest of plants in these experiments may account for the disparity in results.

Kato (7) reported that gibberellic acid increased respiration and water uptake in pea stem segments. Increased cell elongation has also been found following treatment with gibberellins (8). In Earlypak tomato fresh-weight increases seem to be coupled with greater water uptake as well as increases in dry matter content. These increases point to further critical studies of gibberellin effects on the mechanism of water uptake, cell enlargement, and cell division.

Gibberellin profoundly affects the reproductive development of Earlypak tomatoes. While flowering was accelerated only slightly (3 to 6 days) by treatment with gibberellins, this and other papers (5, 10, 11, 12, 13, 15, 17) suggest further studies of the influence of Ga on flowering of various plants. The value of leaf numbers preceding the appearance of flower parts as an index of accelerated flowering has received considerable emphasis (16). It is of especial interest, therefore, that although Ga hastened or did

not affect flowering, it did not influence the number of nodes preceding the appearance of flowers in these experiments. This indicates the need for careful consideration of the plant material when leaf numbers are used as an index of flowering. The implication is that in some plants Ga may accelerate maturation of vegetative parts preceding flowering. This is further emphasized by the induction of flowering in biennial (10) and winter annual plants (5) without cold treatment.

The pronounced fruit-setting ability of gibberellin is of interest both physiologically and economically. The search for new growth regulators to control fruit setting of tomatoes has made fleetingly prominent a variety of chemicals which experimentation and commercial use have proved only partially satisfactory. Aside from their typical formative and other undesirable effects, these compounds are generally effective within a limited range of concentrations below which fruit set is inadequate and above which injury to both fruit and plants frequently results. While it is emphasized that the quality of fruits harvested from sprayed flowers in these experiments was not evaluated critically, similar responses to sprays of 1 to 500 μgm/ml Ga indicate the desirability of continued investigation under temperature conditions unfavorable for fruit set.

Summary

Gibberellin (Ga) stimulated growth, flowering, and fruit set of Earlypak tomato. Total stem elongation of plants, as measured from the cotyledonary node to the node preceding the first flower cluster, was significantly increased by twice-weekly applications of Ga at concentrations of 100 and 200 μgm, but not by concentrations of 25 or 50 μgm per plant. Neither single nor repeated treatments with 25 μgm applied to the expanding leaves at the first, fourth, eighth, or twelfth nodes produced significant stem height differences. Stem elongation of young plants (4 to 6 leaves) was increased, as compared with the control, at concentrations of 2,5, 15, 25, 50, 150, 300 and 450 μgm per plant. Both fresh-weight and dry-matter content of young Earlypak tomato plants were significantly increased. Leaf enlargement, the development of entire instead of incised margins, and an inward rolling of leaflets resulted from treatments with high concentrations of Ga.

Single or repeated applications of Ga at concentrations of 25, 50 and 100 (but not 200) μgm per plant hastened flowering by 3 to 6 days without affecting the number of nodes preceding the first flower cluster. Setting of normal and parthenocarpic fruit was increased by repeated floral sprays of Ga at concentrations from 1 to 500 μgm/ml. Spraying the developing fruit did not increase fruit size.

Appreciation is expressed to Dr. F. H. Stodola, Northern Utilization Research Branch, U.S.D.A., Peoria, Illinois, for supplying gibberellins.

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GROWTH OF THE POTATO TUBER[1,2]

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Early workers, for example Amelung (1) and Sachs (31), considered plant growth to be determined by the number of cells; the larger plant organs had the greater number of cells. Evidence contrary to this theory was presented by Sierp (33), for he observed cell size to vary between similar areas from two plants of the same species or between organs on the same individual. Sinnott (34) found relationships similar to those reported by Sierp between pith cell sizes and dimensions of the petiole in leaves of *Acer saccharum* Marsh.

Loomis (24) defined growth as consisting of cell division and cell enlargement, with cell differentiation as the final phase of development. Typically these processes are completed rapidly, so that only a local region remains meristematic, or, in fruits, division may be general but stop early and subsequent "growth" is then completed by cell enlargement. Houghtaling (19) reported the latter type of growth for *Lycopersicum esculentum* fruit. Cell division was found to proceed from initiation of the ovary to anthesis; from anthesis to maturity, growth of the fruit was by cell enlargement. Sinnott (35) found a relationship between large and small fruited members of the Cucurbitaceae similar to that found in tomatoes. Cell diameters in all tissues in the fruits were found to show little increase from initiation up to anthesis; growth beyond anthesis was completed by cell enlargement alone. The enlargement phase of growth started in the interior tissues first and in the epidermal tissue last. Tetley (37) observed a different type of growth in the apple fruit, with increase in apple tissue after flowering by both cell enlargement and cell division.

The root tips of various plants have been found to be an excellent material for studies of the cellular and chemical changes associated with growth. A root tip cell passes rapidly through the three phases of growth: division, enlargement, and differentiation (3, 8, 12, 16). Baldovinos (3) characterized growth in the corn root tip as dependent upon cell division and a moderate rate of daughter cell enlargement in the first millimeter; a rapid rate of division and elongation in the second millimeter; and cell enlargement only in the third and fourth millimeters. Increase in protein nitrogen per cell was parallel with cell enlargement in the second millimeter of the root tip; rapid but slower than enlargement in the third millimeter; and the protein content per cell decreased in the fourth and fifth millimeters. Although synthesis of protein nitrogen has been reported by others to take place in enlarging cells (5, 15, 29), decrease in protein nitrogen has also been reported (3, 7, 12). The work of Baldovinos (3), Chao (9) and others indicates that both reactions may occur in the same cell at different stages of development.

The morphology and ontogeny of the tuber from *Solanum tuberosum* L. have been studied by Artschwager (2), but little has been done in establishing the cellular changes that accompany tuber development. Esmarch-Bromberg (13) noted that individual pith cell diameters increased fourfold as the tubers increased from 1 to 1.5 cm to maturity. Lehmann (21), using a variety with spherical tubers, examined the relationship between organ size and cell size as the tuber increased in weight. He concluded that increase in cell number and cell size were both concerned in tuber growth. The present work was undertaken to obtain additional information on the growth and chemical changes of the potato tuber as it developed from the stolon tip to a weight of 200 gm.

MATERIALS AND METHODS

Tubers from 50 hills of field grown Cobbler potatoes were dug on each of nine dates during the late spring and summer of 1951. The samples obtained represented tuber development from stolons to 200 gm tubers. The weight of washed tubers was used as a criterion for separating the tubers into different groups representing growth stages. At each stage of maturity, three or four groups were analyzed for chemical composition and two or three groups were analyzed histologically. Samples for chemical analysis were killed and extracted in hot 80% ethanol. To obtain histological material, thin sections were cut parallel to the diameter of the tuber, killed and preserved in formalin–acetic acid solution.

The tuber material for histological analysis was dehydrated in ethyl alcohol (32) and embedded in paraffin. The embedded specimens were cut at 12 or 15 μ, mounted on slides, and stained in fast green.

The size and number of cells per tuber were determined by a modification of the technique developed by Lehmann (21). A prepared slide was mounted in a horizontal projection microscope and the section image projected at a magnification of 210 diameters. A circle of known radius was placed in the projected image with a piece of white paper under the circle, and a check mark was placed in each cell image as it was counted. The total number of cells was obtained in 7 to 10 areas in the pith-storage and

[1] Received revised manuscript May 2, 1957.

[2] Journal paper no. J-2162 of the Iowa Agricultural Experiment Station. Project 1139. This paper is a portion of a thesis, copies of which are deposited in the Iowa State College Library, Ames, Iowa.

cortex tissues of each section. An average cell radius was obtained from the average number of cells in a given circle with the formula:

$$\text{average cell radius} = \frac{\text{radius of circle used}}{\sqrt{\text{average number of cells per circle}}}.$$

The above formula holds if the tuber and each cell within the tuber is assumed to be spherical. The assumption is considered valid, for micrometer measurements of different cell diameters on the same tuber revealed no significant difference, and there was no statistically significant difference between diameter measurements of an individual cell. The cell volume was obtained by applying figures derived from the above formula to the conventional equation for spherical volumes. As the volume was computed from histological material, it was subject to the shrinkages that embedded material undergo. This means that the actual cell volume was larger than reported in this paper. The estimated shrinkage was between 30 and 60 % in volume. As all the material received similar treatment, the cell volume is considered valid for comparative purposes.

The number of cells in the cortex or pith-storage region was determined by dividing the volume of that region by the volume of an average cell from the same region. The volume of the two types of tissue studied was computed in the following manner. External measurements with a micrometer gave the figures used to compute the total volume. The volume of the pith-storage tissue was found by measuring the distance with an ocular micrometer along a diameter from one set of internal phloem units to the other and applying these figures to the formula for volume. The volume of the cortical tissue was the difference between the volume of the entire tuber and the volume of the pith-storage tissue. Although this method of measuring the number of cells in a tuber may not precisely evaluate the exact number of cells present, it gives valid comparisons among different tubers when the cell number of the tubers studied is computed by this technique.

Aliquots of the alcohol extract were evaporated under reduced pressure and soluble nitrogen determined by the semimicro Kjeldahl method (39). The dried residues from the alcohol extraction were ground in a micro Wiley mill to pass a 40 mesh screen, and duplicate 30 to 40 mg samples were used for insoluble nitrogen determinations. The alcohol insoluble nitrogen concentrations were converted to protein by multiplying by 6.25.

Determinations of reducing and nonreducing sugars were made on other aliquots of the alcohol extract. The alcohol was evaporated and the residue diluted with water, cleared with neutral lead acetate, and deleaded with K_2HPO_4 (22). Duplicate determinations, blanks, and known concentrations of glucose were run by the semimicro method outlined by Hassid (17, 18). Sucrose was hydrolyzed with invertase. The difference between values obtained for free reducing and total hydrolyzed sugars was reported as sucrose.

Starch determinations were made on the ground dried material with the method suggested by Powell (28). Samples of the dried ground material were gelatinized under 15 lbs steam pressure for one hour, cooled, inoculated with saliva diluted one to one with water and incubated under toluene overnight at 30° C. The solutions were then cleared and deleaded as previously described. Hydrolysis of the saliva digest was completed with HCl, and duplicate determinations of reducing sugars were made by the ceric sulfate method.

The remaining dried residues of each weight group were combined into one sample. One-half of the sample was weighed into a tared, fritted glass cylinder and hydrolyzed with 0.56 *N* HCl for one hour at 15 lbs pressure (25). The washed, dried residue from the hydrolysis was reported as wall material. The remaining half of the sample was weighed into tared crucibles, heated in an electric muffle furnace at 500° C until all evidences of carbon were removed, and the residue reported as ash.

Tuberization and Growth

Stolon formation in the potato begins at the lower nodes near the seed-piece and progresses upward. Stolons arise from axillary or sometimes from auxiliary buds and grow outward, or outward and downward. The first tubers usually form on the lower stolons, and these tubers tend to become dominant, forming the largest potatoes. The general picture is one of a downward polarity of stolon and tuber formation.

Tuber formation shows first as a thickening of the first internode back of the stolon's apical bud.

Table I

Number and Size of Cells in Potato Tubers

Fresh wt of tubers, mg	Cells per tuber (10^6)	Pith-storage cell volume, $mm^3 \times (10^{-4})$	Cortex cell volume, $mm^3 \times (10^{-4})$
22 *	0.149 ...	0.82 ...	0.20 ...
37	0.375 ± 0.23	0.94 ± 0.08	0.77 ± 0.07
107	0.919 ± 0.15	0.85 ± 0.13	0.71 ± 0.20
140	1.27 ...	0.82 ...	0.83 ...
193	1.62 ± 0.01	1.62 ± 0.15	0.67 ± 0.19
500	2.02 ± 0.58	1.79 ± 0.09	1.18 ± 0.07
875	3.17 ± 0.15	1.58 ± 0.02	1.31 ± 0.13
1,013	3.85 ± 0.14	1.68 ± 0.15	1.05 ± 0.07
2,007	6.98 ± 0.08	2.04 ± 0.87	1.58 ± 0.10
5,065	18.6 ± 0.19	2.98 ± 0.08	1.59 ± 0.06
10,384	19.0 ± 0.95	3.17 ± 0.14	1.49 ± 0.10
19,987	38.4 ...	3.52 ...	2.17 ...
49,942	77.3 ± 0.74	4.11 ± 0.11	2.40 ± 0.18
74,363	148.1 ± 0.09	4.33 ± 0.10	2.30 ± 0.07
100,125	118.1 ± 0.07	6.40 ± 0.15	3.27 ± 0.08
149,500	121.1 ± 0.14	9.17 ± 0.39	5.32 ± 0.30
200,300	183.1 ± 0.16	9.81 ± 0.70	6.34 ± 0.82

Averages of counts on two or more tubers in each size class. The standard errors of the replicate means are given.

* Stolons.

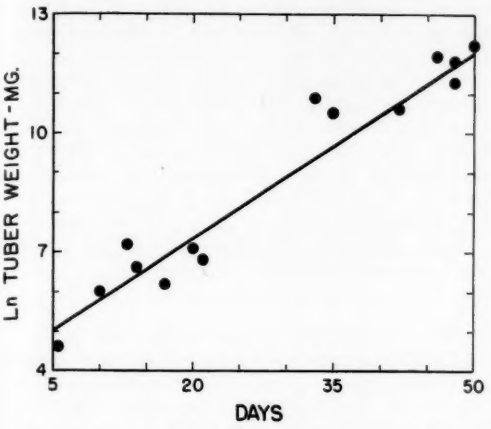

FIG. 1. The relationship between time in days and tuber wt in gm. The natural logarithm of tuber wt is plotted as a function of time. The line was fitted by method of least squares.

This thickening depends upon growth in the cortical, pericyclic, vascular, and pith parenchyma (2), rather than upon a normal cambial development. The data of table I show that cell number was increasing, and therefore cell division was continuing, in tubers up to 200 gm, which is above average marketable size. As the tuber increased in size from 37 mg to 200 gm there was 500-fold increase in cell numbers, and a corresponding 10-fold increase in the average size of individual cells. The continuous increase in cell number, table I, indicates that cell division was taking place during the entire period of tuber growth.

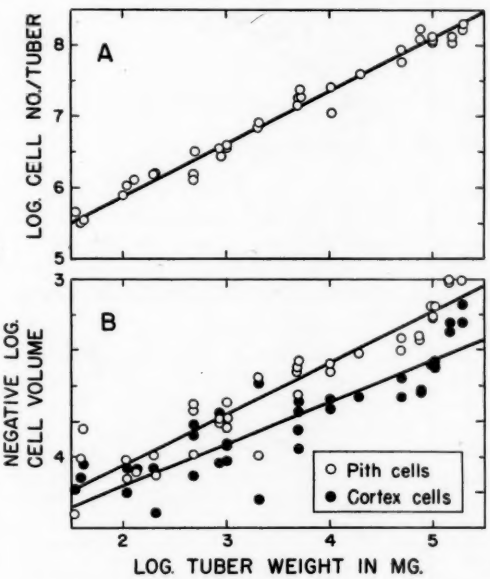

FIG. 2. (A) The relationship between number of cells/tuber and tuber wt in mg when plotted on a log-log scale. (B) The relationship between volume of pith-storage or cortex cells and tuber wt when the log of vol. in ml is plotted as a function of the logarithm of tuber wt in mg. The lines were fitted by the method of least squares.

Blackman (4) has proposed that during the active period of growth of an organism the relationship between increase in size and time can be expressed by the equation:

$$W = W_o e^{rt} \qquad (1)$$

Although many methods can be used to measure growth, an increase in fresh weight is used to indicate growth in this experiment. W is the fresh weight at time t, W_o is the initial fresh weight, and

TABLE II

A SUMMARY OF THE ALLOMETRIC COEFFICIENTS AND THE RELATIVE DAILY INCREASE IN CELL NUMBER AS WELL AS FACTORS ASSOCIATED WITH GROWTH AND DIFFERENTIATION OF THE POTATO TUBER

	"K" OR ALLOMETRIC COEFFICIENTS *	FIDUCIAL LIMITS, 5 % LEVEL	"KR" OR THE RELATIVE RATE OF DAILY INCREASE, %	FIDUCIAL LIMITS, 5 % LEVEL
No. cells/tuber	0.73	± 0.03	11.5	± 0.8
Vol. cortex cells	0.23	± 0.04	3.6	± 2.1
Vol. pith-storage cells	0.28	± 0.03	4.4	± 0.8
Fresh wt/cell	0.26	± 0.14	4.1	± 1.0
Insoluble N (×6.25) as protein/cell	0.24	± 0.05	3.8	± 0.9
Wall material/cell	0.25	± 0.08	3.9	± 1.4
Dry wt/cell	0.30	± 0.05	4.7	± 1.0
Nonreducing sugars/cell	0.34	± 0.08	5.3	± 1.5
Starch/cell	0.35	± 0.05	5.5	± 1.2
Soluble N/cell	0.32	± 0.19	5.0	± 2.9
Ash/cell	0.31	± 0.13	4.7	± 2.0

* All allometric coefficients were significant above the 1 % level when tested for significance by the t test.

r is a constant that is often termed the relative growth rate (6, 14, 38). This same equation expressed in the logarithmic form

$$\ln W = \ln W_o + rt \qquad (2)$$

describes a straight line. Therefore if the natural logarithm of tuber weight is plotted against time, a straight line can be fitted to the data by the method of least squares. The numerical value for r or the relative growth rate is the slope of the line in figure 1. Under conditions of this experiment the value for r is 0.16 ± 0.01 day^{-1} or 16 % per day. The deviations of the points from the line in figure 1 are caused by the variation found in tuber weights at any sampling date. The figures used to plot the line were obtained by averaging the weights of all the tubers dug at any sampling date. Although a controlled laboratory experiment would give a more precise measure of the relative growth rate, the figure

of 16 % per day is valid for the materials and conditions.

Huxley (20) and others (23, 30) have demonstrated that the relation between individual components either chemical or morphological to the organism as a whole follows the pattern termed allometric (20) and expressed

$$Y = CW^k \qquad (3)$$

The symbols in this equation indicate arbitrary values. Y could be number of cells, cell volume per tuber, mg of protein per cell, mg of starch per cell or other morphological or chemical cellular constituents of the potato corresponding to the tuber weight W. C is a constant. The exponent k is what Huxley (20) terms the allometric coefficient. This k expresses the ratio of the relative rate of increase in the component being studied to the relative rate of increase in the whole body of the organism, or in the case of the tuber, increase in weight. The logarithmic form of equation *3*

$$\ln Y = \ln C + k \ln W \qquad (4)$$

describes a straight line. The value for k is the slope of the line formed when $\ln Y$ is plotted against $\ln W$. Figures 2 A, B; 3 A, B, C, D; and 4 A, B, C show such relationships. In each case the line is fitted to the data points by the method of least squares. Although using the method of least squares on logarithmic data may induce some distortion, it is not believed important enough to consider weighing the data points. The values for k or the allometric coefficients corresponding to the cell number, cell volumes, or different chemical constituents for which the tuber was analyzed are found in table II. An example of a typical allometric equation expresses the relationship between cell number and tuber weight.

$$Y = 3.71(10^4)\ W^{0.73} \qquad (5)$$

The value for k was computed as described above.

The daily increase of the morphological and chemical constituents in growth of the tuber can be determined by the relationship in tuber weight that exists between equation and *1* and *3*. By substituting W in equation *3* for its equal from equation *1* the following expression is derived

$$Y = Ce^{krt} \qquad (6)$$

As all the components of equation *6* have been determined, the daily increase can be computed by mul-

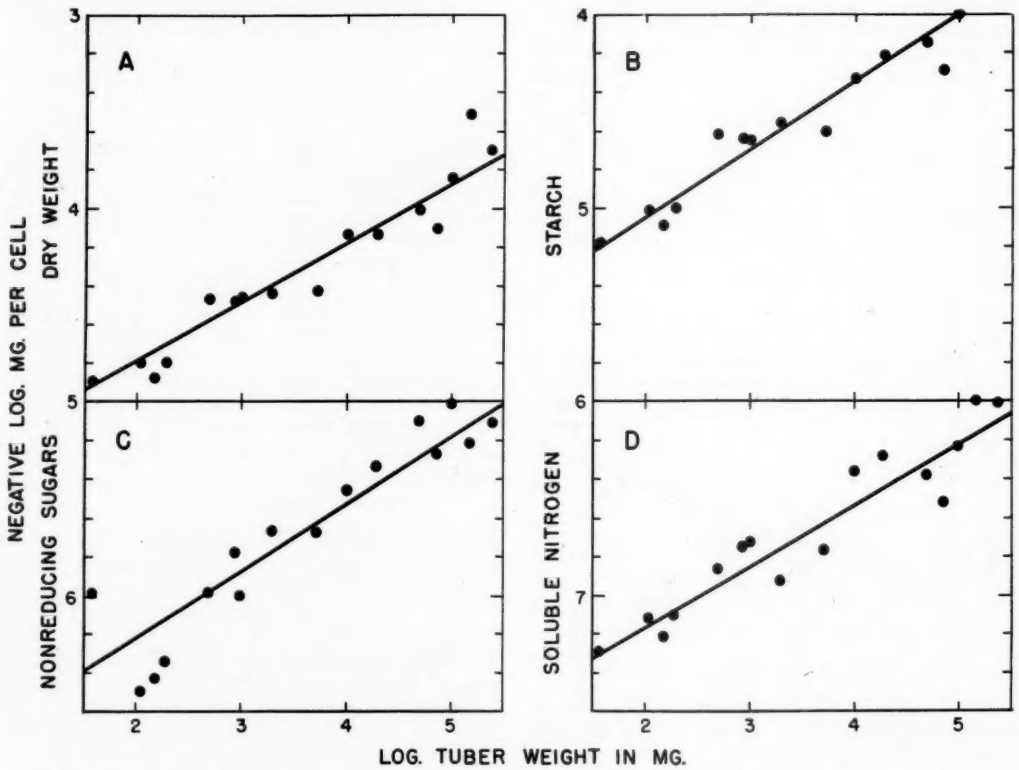

FIG. 3. The relationship between (A) mg dry wt/cell, (B) mg starch/cell, (C) mg nonreducing sugars/cell, and (D) mg of soluble nitrogen/cell when plotted as a function of tuber wt in mg on a log-log basis. The lines were fitted by the method of least squares.

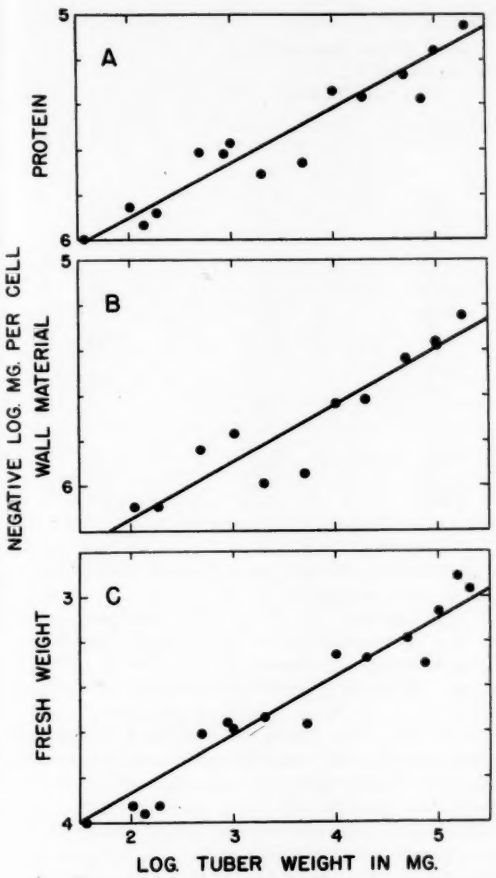

FIG. 4. The relationship between (A) mg of protein, alcohol insoluble N times 6.25/cell, (B) mg wall material/cell, and (C) mg fresh wt/cell when plotted as a function of tuber wt in mg on a log-log basis. The lines were fitted by the method of least squares.

tiplying the values for k with that for r. For example, the daily increase in cell number per tuber is (0.16) (0.73) or 11.7 % per day. The daily increase in cell volume or chemical constituents is listed in table II. This type of allometric analysis differs from that which was reported several years ago by Huxley (20) and others in that the tuber growth data is used throughout in calculating the rk values, for example, the actual time rates are computed.

Chemical Changes

The components for which the developing tubers were analyzed may be divided into three groups on the basis of the allometric coefficients shown in table II. The allometric coefficient of 0.73 for cell number is the highest. A group of components usually associated with tissue differentiation such as dry weight, nonreducing sugars, starch, alcohol-soluble nitrogen, and ash had allometric coefficients averaging 0.32. The smallest allometric coefficients were associated with factors such as cell volume, fresh weight, protein nitrogen, and wall material. These latter factors are more closely associated with meristematic tissue than differentiating tissue.

The allometric coefficients associated with the chemical constituents were derived in a manner similar to those for cell number. For example, figures 3 A, B, C, D show the relationship between the gain in tuber weight and the gain in dry weight, starch, nonreducing sugars and soluble nitrogen per cell. The slope of the respective curves is the allometric coefficient or the ratio of the relative rate of gain in tuber weight to the relative rate of gain of various constituents associated with tissue differentiation. The quantity of ash per cell also increased as the tuber gained in weight (table III). The type of curve generated when the milligrams of ash per cell are plotted as a function of tuber weight on a log-log scale is similar to those shown for other factors associated with tissue differentiation. The k value of 0.31 falls within the group of those allometric coefficients asociated with differentiation factors (table II). A similar set of curves, seen in figures 4 A, B and C, relates the factors associated with meristematic tissue, and tuber weight. The "allometric coefficients" of these meristematic factors are summarized in table II.

Plotting the factors associated with tissue differentiation and meristematic tissue in cellular units as a function of tuber weight on a log-log basis brings out one important consideration that is often obscured in linear plotting. As the tuber increases in weight, there is a corresponding increase in the different constituents, but they increase by a constant multiplication rather than by addition. This is brought into focus if we consider that equal divisions on a logarithmic scale denote equal amounts of multiplication whereas equal spaces on an absolute scale denote equal additions.

Reducing sugars, computed as milligrams per cell unit, fluctuated over a wide range of values as seen in table IV. This fluctuation may be related to the amount of illumination or to other ambient conditions. The residue from the HCl hydrolysis, considered to be wall material, increased in weight per cell unit as the tuber increased in size, as seen in figure 4 B, but table IV indicates the wall material remained constant when computed as mg per μ^2 of cell surface, showing that no secondary wall thickening had occurred in 200-gm potatoes.

Discussion

Observations on field grown potato plants showed that the first stolons were formed on the lower nodes, with production on higher nodes as the season progressed. Stolons have been observed by Page (27) to arise in acropetal succession as the plant develops, suggesting that the subterranean nodes must attain a

TABLE III

FACTORS ASSOCIATED WITH DIFFERENTIATION OF THE POTATO TUBER (EXPRESSED IN CELLULAR UNITS)

FRESH WT TUBERS, MG	ALCOHOL SOL N/CELL, MG × (10^{-6})	NONREDUC SUG/CELL, MG × (10^{-6})	STARCH/CELL, MG × (10^{-5})	DRY WT/CELL, MG × (10^{-4})	ASH/CELL, MG × (10^{-6})
22	1.14	0.74	0.56	0.16	...
37	0.51	1.04	0.64	0.13	...
107	0.72	0.31	0.97	0.16	...
140	0.58	0.36	0.81	0.13	...
193	0.74	0.44	0.96	0.16	0.22
500	1.39	1.04	2.30	0.34	0.80
875	1.67	1.68	2.25	0.33	...
1,013	1.77	0.93	2.22	0.34	0.69
2,007	1.20	2.11	2.66	0.36	0.62
5,065	1.68	1.80	2.51	0.37	1.23
10,384	4.21	3.51	4.44	0.67	1.00
19,987	5.23	4.54	5.98	0.67	0.69
49,942	4.03	7.69	6.99	1.00	1.83
74,363	3.02	5.30	5.04	0.76	...
100,125	5.78	7.99	10.16	1.42	3.03
149,500	9.89	5.93	20.67	2.55	...
200,300	8.77	7.32	14.19	1.99	3.56

certain stage of development before stolons are initiated. The greatest percentage of stolons that produced large tubers were located on nodes near the seed piece. Clark (10) observed a tendency for the upper stolons to produce smaller tubers and for tubers that grew to exceed half an inch in diameter to be those formed at the first of the season. The older stolons which tuberized first may have exerted such an influence in obtaining the factors needed for tuber growth that younger tubers could not overcome this competition. Page (27) observed that the growth rate of tubers initiated at approximately the same time was unequal, and growth of quiescent tubers could be started by removal of older tubers. The cooler soil temperature which prevailed around the deeper tubers near the seed piece may have aided in tuberization or growth after initiation.

Tuber growth is the result of increase in cell number and increase in cell size. Unlike the fruit of *Lycopersicum esculentum*, which was found by Houghtaling (19) to have an initial period of cell multiplication followed by a period of enlargement of the existing cells, the potato tuber showed enlargement accompanying cell multiplication throughout the range of tuber weights studied, from less than 50 to more than 200,000 mg. This result is in agreement with those reported by Lehmann (21), who concluded that growth of spherical tubers is accompanied by mitotic activity proceeding with equal rates in all directions, and an accompanying increase in cell size.

TABLE IV

FACTORS ASSOCIATED WITH GROWTH OF THE POTATO TUBER (EXPRESSED IN CELLULAR UNITS)

FHESH WT TUBERS, MG	INSOLUBLE N AS PROTEIN/CELL, MG × (10^{-6})	FRESH WT/CELL, MG × (10^{-4})	REDUC SUGARS/CELL, MG × (10^{-6})	WALL MATERIAL/CELL, MG × (10^{-6})	WALL MATERIAL/μ^2 CELL SURFACE, MG × (10^{-10})
22	2.31	1.48	2.22	...	...
37	0.98	0.99	1.04	...	...
107	1.40	1.16	1.12	0.81	0.85
140	1.18	1.10	0.74	...	...
193	1.32	1.19	1.12	0.81	0.85
500	2.48	2.48	2.27	1.44	1.15
875	2.42	2.76	2.29	...	...
1,013	2.71	2.63	2.82	1.82	1.46
2,007	1.95	2.87	2.98	1.00	0.62
5,065	2.22	2.73	2.38	1.11	0.55
10,384	4.58	5.48	4.10	2.29	1.04
19,987	4.24	5.21	2.40	2.40	1.01
49,942	5.37	6.46	3.12	3.62	1.39
74,363	4.20	5.03	2.69	...	...
100,125	6.94	8.46	2.73	4.24	1.24
149,500	10.74	12.37	1.92	...	...
200,300	8.91	10.97	2.37	5.67	1.19

In order for an organ to grow and to maintain the same relative size relationship of its different members to the organ as a whole, the allometric coefficients of all the members would have to be unity (20). When the allometric coefficients are not, the different members of the organism grow disproportionately. The potato tuber follows the system of disproportionate growth. The high allometric coefficient of 0.73 for increase in cell number, approximately three times that for cell volume, indicates that tuber size was more directly related to an increase in cell number than an increase in cell volume. This fact was also substantiated when comparing a 37-mg and a 200-gm tuber. There was an increase of approximately 500 times in cell number with an increase of only 10 times in cell volume.

The allometric coefficient for increase in volume of cortex is smaller than pith-storage tissue. The higher rate of volume increase for the pith-storage cells predicts that eventually the pith-storage tissue will make up the bulk of the tuber. A comparison of small and large tubers shows that cortex tissue decreased from 50 to 15 % as the tuber ages.

The increase in cell number as the tuber increases in size indicates that cells, presumably vacuolated cells, are dividing. The slow rate of cell enlargement suggests that daughter cells have to reach a size slightly greater than the parent cell before they undergo division. Sinnott (35) observed that cells in certain cucurbit tissue divided in this manner. Sinnott and Bloch (36) also reported divisions in vacuolated cells 50 to 100 μ in diameter; cells of this diameter are within the range of those observed for the potato tuber.

Although more wall material was laid down as the cells increased in size, the weight per μ^2 of wall surface remained constant, which is characteristic of meristematic cells, and indicates the absence of secondary wall formation. O'Kelley and Carr (26) found no secondary thickening in the wall of the cotton fiber during the elongation period of two to four weeks after anthesis, whereas the later period of fiber development consisted mainly of secondary wall formation. The 200-gm tubers, which were the largest observed, had not completed growth, and it was not possible to determine whether there might be some secondary wall formation in a larger tuber.

Growth of the potato tuber can be characterized by a rapid accumulation of growth materials such as protein, and of storage or differentiation materials such as starch or sucrose. Over the growth period studied, cell volumes, green weight, protein and cell wall material per cell unit increased steadily, about 4.0 % per day. Dry weight, nonreducing sugars, starch, alcohol-soluble nitrogen and ash per cell increased more, about 5.0 % per day. The first group of factors would be more closely associated with growth, and the second with maturation or differentiation of tissue. The maturation factors were low in absolute quantities per cell at first (tables III and IV), but their more rapid increase would make them dominant in the 200-gm tuber. The failure of the cell wall to increase in weight on a cell surface area basis has been discussed as evidence of a meristematic tendency in the potato tuber. The accumulation of protein is the characteristic process of cell division and of some aspects of cell enlargement (3, 9, 15). These trends indicate that the normal tendency toward growth followed by differentiation was present, although surpassed by the remarkable meristematic activity of the potato cell.

The equation derived for the relationship between increase in tuber weight and time follows the equation proposed by Blackman (4) instead of a sigmoidal type of growth. The type of growth that follows the Blackman equation would be expected of normal meristematic tissue. It is possible that a sigmoidal growth curve would have been obtained if this work had included tubers larger than 200 gm.

The development process in plants has been divided by Loomis (24, p. 241) into growth and differentiation. In this reference he defines growth and differentiation as, " . . . the increase in size due to cell division and cell enlargement, and differentiation as the sum of the chemical and morphological changes which start during cell enlargement and end with the death of the cell." The potato tuber did not follow the expected growth pattern, for cell division, as well as enlargement and differentiation, were progressing simultaneously over the range of tuber weights studied. From the smallest tubers distinguishable from the stolon to the largest studied, protein per cell increased almost as rapidly as cell volume—9 times against 10.

All of these considerations indicate that the potato tuber, if not unique, is certainly unusual in its growth pattern. Here is the phenomenon of a maturing storage tissue, well filled with starch, which is still meristematic in its cell wall and protein characteristics, with its cells still dividing and enlarging. One might suspect that a growth of this sort is dependent upon unusual concentrations of growth materials and a liberal supply of growth hormones.

Summary

Field grown potato tubers varying in size from stolon tips to 200 gm were analyzed histologically and for different chemical components. A technique is presented for measuring cell number and cell size in tubers of varying weight.

The number of subterranean nodes producing stolons on the potato plant increased upward as the plant became older, but the largest tubers were produced on the lower, older stolons of the plant.

The allometric relationships between number of cells, the volume of the individual cells and tuber weight indicate that cell multiplication was the factor most responsible for increase in size of the tuber. The number of cells per tuber increased 500 times as the potato increased from 37 mg to 200 gm, while cell volume increased only 10 times.

Factors considered to be associated with growth,

such as volume of cortical and pith-storage cells, green weight, wall material, and protein are expressed as milligram per cell unit. A similar set of factors usually associated with tissue differentiation or maturity such as starch, soluble-nitrogen, ash, dry weight, and nonreducing sugars are also expressed as milligrams per cell unit. When concentration per cell of the individual growth or differentiation factors are plotted as a function of time on a log-log scale, a straight line is obtained. The slopes of the respective lines gave the allometric coefficients. These allometric coefficients indicate that the factors associated with differentiation or maturity are increasing in the cell at a faster rate than growth factors. Although the differentiation factors were present in low concentration initially, they increased rapidly in concentration and became the dominant constituent in the cell.

The potato tuber is exceptional among plant tissues in its long continued meristematic development. Almost uniform rates of cell division and cell enlargement appeared to be maintained in the pith and cortical tissues between 2-gm and 200-gm tuber weights, even though the cells of the larger tubers had accumulated the storage materials characteristic of mature tissues.

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ACTION SPECTRA OF PHOTOMORPHOGENIC INDUCTION AND ITS PHOTOINACTIVATION[1,2,3]

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Photomorphogenesis is concerned with the regulation of plant growth by visible radiant energy. The term is applied here only to those reversible photochemical reactions which are most actively excited at the red end of the visible spectrum and exert control of such diverse processes as seed and spore germination, seedling development and flowering. The kinetical similarity of these responses in regard to their action spectra, logarithmic response to the incident energy, low level of threshold energy required to induce the reaction, and the capacity of the induction process to be reversed or blocked by far-red energy, have led to the general conclusion that they are all controlled by the same photochemical mechanism and involve the same photoreceptor pigments. The spectral region of maximum effectiveness for induction has been shown to be in the red between 600 and 700 mμ (2, 4, 9, 16, 17, 18, 19, 25) and the consequences of red induction can be prevented by subsequent or simultaneous irradiation with far-red between 700 and 750 mμ (3, 6, 7, 10).

The action spectrum of the photocontrol of seedling development has been studied in relation to the following responses: a) inhibition of the first internode in Avena (1, 9, 20) and several other grasses (21); b) stimulation of the Avena coleoptile (1); c) stimulation of leaf growth in pea and bean (19, 15); d) inhibition of the hypocotyl of bean; and e) stimulation of epicotyl stem and leaf growth in bean (11, 12, 25). The action spectrum of seed germination and spore germination have shown similar general characteristics (4, 5, 16). The responses of the seedling organs thus far studied are directly proportional to the logarithm of the incident energy (12).

These different organs were all considered as potential assay systems for the action spectrum studies. The Avena first internode is the classical organ which has been most extensively investigated, but its extreme sensitivity makes it difficult to handle and results often are not very uniform. The straight hypocotyl portion of the stem of the Black Valentine bean also is quite sensitive. In addition, the excised hypocotyl does not lend itself well to growth studies since the base tends to split. It also is relatively difficult to measure straight growth as compared with the measurement of a stem angle. The hypocotyl hook of bean is much less sensitive and, with a 20-hour light exposure, requires approximately 1000 times as much energy for threshold activity as the first internode of Avena. In view of these considerations, the hypocotyl hook was selected as the most suitable organ for the precise determination of the action spectrum of photomorphogenesis. Its sensitivity is sufficiently high for use with low intensity monochromatic sources and our experience with it has shown that it gives very reproducible results. It is large enough to be handled easily and the hook angle can be quickly read to the nearest 5 degrees.

MATERIALS AND METHODS

BIOLOGICAL MATERIALS: The same general technics were used as previously described for the hypocotyl hook bioassay (11). Dry seeds of *Phaseolus vulgaris*, var. Black Valentine, sieved for uniform size, were germinated and grown in gravel beds subirrigated with tap water in complete darkness in a constant condition room maintained at 25° C and 75 % relative humidity. At 6 days from seeding, the hooks were selected for a zero degree angle and arranged in a circle on moist filter paper in a 150-mm Petri dish, 20 hooks per dish. Manipulation of the hooks was done over a green safelight table with a second safelight mounted above the table. No measurable hook opening response to the safelight was observed with an exposure period of 1 hour (23). The experimental safelight exposure actually never exceeded 10 minutes and was usually much shorter. Each experiment was terminated 20 hours after excision with the reading

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of the hook angles, using a parallel-line grid protractor calibrated in 5-degree units.

IRRADIATION: The monochromatic irradiation system consisted of ten interference-filter monochromator units mounted on wood cabinets as previously described (22). The cabinets were arranged on a long table in a constant condition room. Each monochromator unit contained two Bausch & Lomb, 2-inch square interference filters in tandem, a dyed-gelatin filter of the Wratten type mounted in between, and a 10-cm aqueous filter. An additional aqueous filter of 2 cm of 30 % copper sulfate solution was placed over the exit aperture of the monochromator unit when working in the green and blue in order to completely exclude all red energy that might leak around the filter system. When irradiating with the far-red, a dyed-gelatin supplemental filter was used to absorb the red-induction region shorter than 700 mμ. A spectral range from 365 to 800 mμ was covered in three series of experiments, with at least one wavelength station overlapping between contiguous series. The stations were 10 mμ apart through the red and far-red and approximately 20 mμ in the remaining portions of the spectrum.

The incident irradiances were adjusted to the proper value with a thermopile operating into a recording modulation amplifier. The thermopile was calibrated frequently against a Bureau of Standards standard lamp. Irradiances of 1 μw/cm² or higher, were balanced directly with the thermopile-amplifier system.[4] Lower irradiances were obtained by first adjusting to an intensity of 1 μw/cm² and reducing by half-log factors with neutral density filters. Since dyed-gelatin neutral density filters are not spectrally neutral, especially at the ends of the visible spectrum, it was necessary to make further correction by adjusting the voltage on the incandescent lamp source or, in the case of the mercury arc, the resistance in series with the lamp (24). A photomultiplier detector was used to establish the proper ratio with neutral density filters. This method made it unnecessary to depend upon the nominal density values of the neutral filters or upon the spectral calibration of the photomultiplier tube in order to establish the correct values of irradiance.

The photoreversal series required an induction treatment before subjecting the hooks to the monochromatic irradiation. This was supplied in a separate cabinet containing a red gelatin filter and a bank of 15-watt special fluorescent lamps containing a magnesium arsenite phosphor, which was supplied by the General Electric Company. These lamps emit most of their energy in the red, with a spectral peak at about 660 mμ and relatively little energy longer than 700 mμ. This source had a wavelength band of 600 to 700 mμ and gave an irradiance of 300 μw/cm². The induction exposure time required for an incident energy of 250 mj/cm² was 14 minutes.

[4] One microwatt (μw) in MKS units is equivalent to 10 erg/sec in cgs units; the microjoule (μj) is equivalent to 10 ergs.

CONTROLS: Two controls were used in all induction experiments and three in the inactivation series. For induction, there was a dark control and a standard red control which received 20 hours continuously of 0.1 μw/cm² or a total incident energy of 7.2 mj/cm². In addition to these two, a third control of 14 minutes red, totaling 250 mj/cm² was used for all inactivation experiments. At the end of the 20-hour period, the average dark control angle of opening was zero degrees, the range of the standard red control was 67° to 73°, and for the 14-minute red control was approximately 50°. A deviation from these results was considered as an indication that the hooks were not responding normally. This seldom occurred, but occasionally, because of variations in cultural conditions or failure of the irradiation equipment, the controls did not give standard values; the whole series of results was then rejected.

A separate cabinet and source system was used for the standard red irradiation. The source was a 120-volt incandescent lamp operating at ca 80 volts or 67 % of rated voltage. At this low voltage, an incandescent lamp rated at 1000 hours has over 100 times the life expectancy of a similar lamp operating under normal conditions (24). Consequently, the intensity of the source changed very little with time and a single adjustment of the standard irradiance was sufficient for at least one year of almost continuous use. The filters for the standard cabinet consisted of 10 cm of 1 % hydrated copper sulfate and a red dyed-gelatin filter which, together, yielded a transmission band of approximately 625 to 700 mμ. The source used in the 14-minute red control was similar to that described previously for the reversal series.

In both the induction and inactivation (reversal) experiments, the irradiation periods were kept short as compared to the total experimental time of 20 hours. Where growth is used as the criterion of a photoreaction, the response is the result of many relatively slow thermochemical (dark) reactions which are altered in rate by the photoreaction. Kinetical separation of the two groups of processes can be effected only if the photoreaction time is small compared to the dark time. Also, the monochromatic irradiation involved high-powered lamp equipment spaced over a considerable distance in a constant condition room, thus making it difficult to ensure exactly the same environment for all of the wavelength stations. By making the irradiation time less than three hours, it was convenient to remove the hooks from the monochromator cabinets to a single dark cabinet for the remaining development period of 17 hours.

The irradiation time was a constant value for each group of experiments. It was 2.78 hours for the induction series. For the inactivation series, 14 minutes was used for induction and 30 minutes for inactivation. The induction reaction of various photomorphogenic seedling responses, including that of the opening of the hook (11), is proportional to the logarithm of the incident energy. Reciprocity does not hold for

these processes over long exposure periods. A continuous irradiation of 20 hours (10 μj/cm^2 threshold) is more effective than a 2.78-hour exposure (30 μj/cm^2 threshold) for the same total incident energy. While the photoreversal process, calculated as an inactivation factor, is a linear function of irradiance for incident energies yielding up to about 90 % reversal, it does not follow reciprocity, i.e, the degree of reversibility for a constant value of energy increases with time up to between 1 and 2 hours (13). However, almost complete reversibility can be obtained by the end of the first 30 minutes. Because of the logarithmic function in induction and lack of reciprocity of both of these reactions, results with different incident energies, therefore, are comparable only if all irradiation times are kept constant.

With an interference-filter monochromator system it is possible to establish either a constant incident energy or quantum intensity spectrum for each series. Since a quantum intensity spectrum would have required an energy correction factor at each wavelength, it was more convenient to use a constant incident energy for each wavelength series and then convert to quantum values for the calculation of the action spectrum.

Treatment Schedule: The complete sequence of manipulations for obtaining the induction action spectrum were: 1) Selection and excision of uniform hypocotyl hooks and arrangement in a Petri dish, using a green safelight. 2) Exposure to the appropriate wavelength of monochromatic radiant energy in a cabinet for 2.78 hours (10,000 seconds), this time yielding an incident energy of 10 mj/cm^2 or 100,000 ergs/cm^2 for an incident irradiance of 1 μw/cm^2. The incident energies were adjusted on a logarithm scale of 0.5 log units or a ratio of about 0.3 between energy steps and were 0.1, 0.3, 1.0, 3.0, 10.0, 30.0 and 100 mj/cm^2. 3) Transfer of all hooks to a dark cabinet for a development period of approximately 17 hours to make a total time from excision of 20 hours. 4) Reading of the hook angles with a protractor.

The sequence of manipulations for obtaining the photoinactivation or reversal action spectrum were: 1) Excision of the hooks as above. 2) Induction with energy from a red fluorescent lamp (600 to 700 mμ) for 14 minutes, which yielded an incident energy of 250 mj/cm^2. 3) Transfer for photoinactivation exposure to a monochromator cabinet for a 30-minute period at a specified wavelength and irradiance. 4)

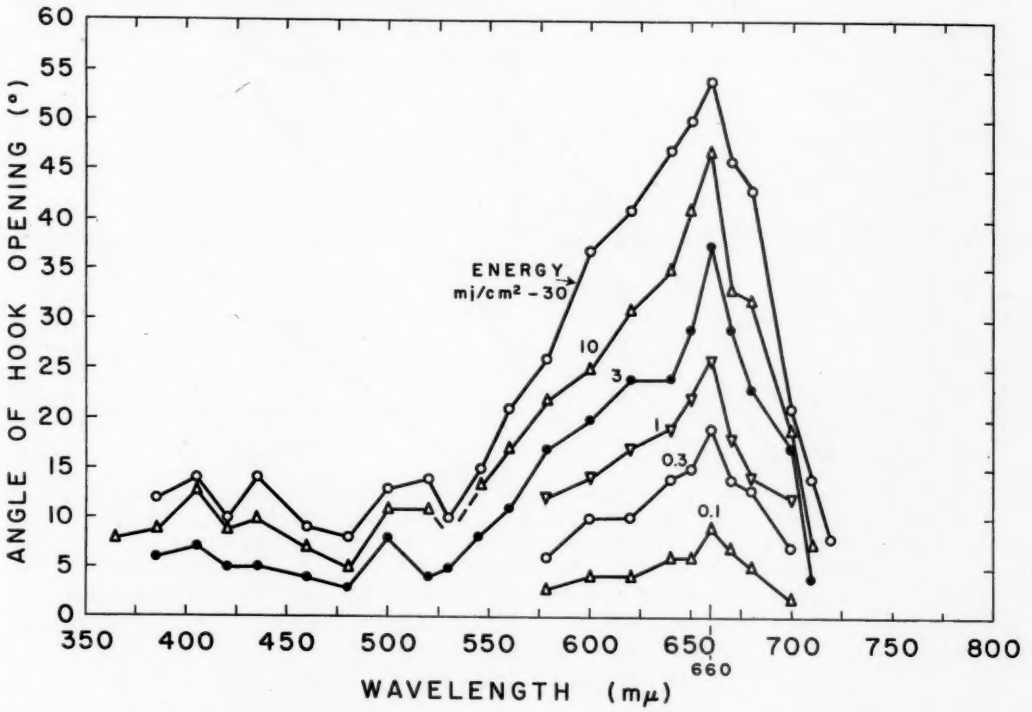


Fig. 1. Equal energy curves for photomorphogenic induction, hook angle vs wavelength, for incident energies of 0.1 to 30 mj/cm^2. The energy was applied over a period of 2.78 hrs (10,000 sec) at 0.01 to 3.0 μw/cm^2. Each curve represents a response to wavelength at one energy level and each point represents the average of 4 to 6 replicates of 20 hooks each.

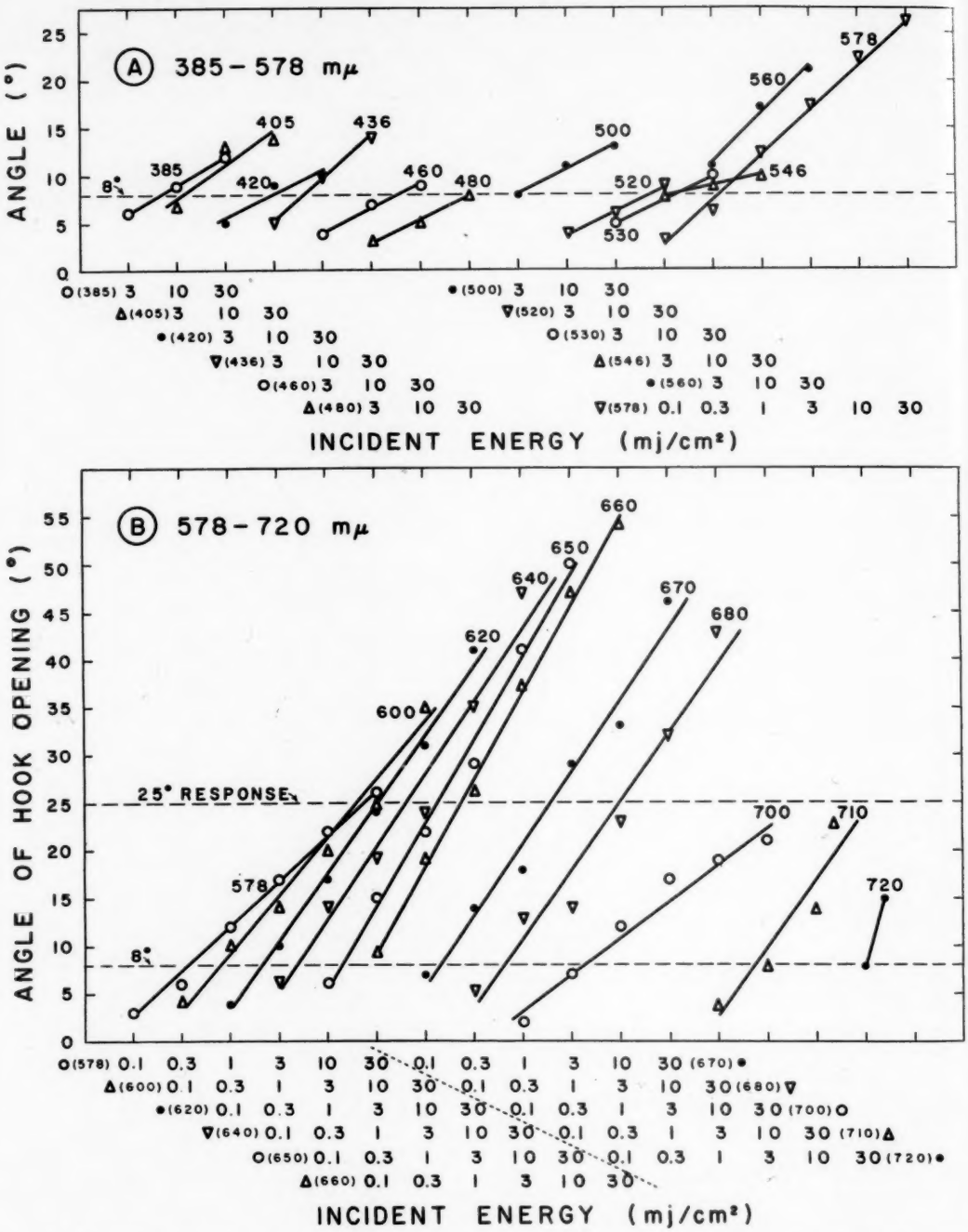


FIG. 2. Linear regression lines of induction, hook angle vs incident energy. Each line represents one wavelength. These graphs were plotted from the same data as in figure 1 for determining the energy required for specified response values. The horizontal dashed lines at the 8° and 25° levels were used for calculation of the induction action spectrum. The energy scales have been shifted one step to the right for each regression line to prevent superposition for the different wavelengths; each curve is represented by a separate scale.

Development in the dark for approximately 19 hours to total a 20-hour period from excision. 5) Reading of the hook angles.

Results

Induction Spectrum: The data for the angles of hook opening were averaged for the various replicates (a minimum of 4 for each point) and a family of equal energy curves plotted as a function of hook angle vs wavelength, figure 1. These curves do not have the same shape as the true action spectrum because they involve equal incident energy instead of equal response and the logarithmic character of the response tends to reduce the relative magnitudes, although not the positions of the peaks.

The data of figure 1 are replotted in figure 2 as a family of wavelength curves of hook angle vs log energy. These curves are straight lines for hook angles above about 5°. In order to prevent the superposition of the curves, each line and its respective energy scale are shifted one unit to the right. The curves were calculated as regression lines by the method of least squares. The shorter wavelengths from 365 to 578 mμ are presented in figure 2 A and the longer wavelengths from 578 to 720 mμ in figure 2 B.

From the equations of the angle vs log energy curves, the incident quanta required at each wavelength to produce an opening angle of 8° and of 25° were calculated. An angle of 8° was the largest that could be obtained throughout most of the spectrum and required a minimum of extrapolation; 25° was about the mid-range of the angles obtainable in the red with incident energies up to 10 mj/cm². The 25° angles were therefore calculated only for the red end of the spectrum since it was not possible to obtain large angular openings in the shorter wavelengths. The action spectra plotted in figure 3 are given as relative quantum responsivity. By multiplying the ordinant by a factor of 0.17, the values may be converted to absolute quantum responsivity in degrees/(nano-einstein/cm²). The nano-einstein is used here because it yields conveniently-sized values. It is 10^{-9} (milli-micro) einstein or mole of quanta. The factor 2.8×10^{-16} converts the data to degrees/(quanta/cm²).

The following summarizes the calculations and formulas used for the induction action spectrum:

a. Calculation of the angle vs log-energy regression lines in the form:

$$(1) \quad \theta = m \log E + b$$

where, θ is the angle of the hook, in degrees; m is the slope parameter; E is incident energy, mj/cm²; and b is the ordinate intercept parameter.

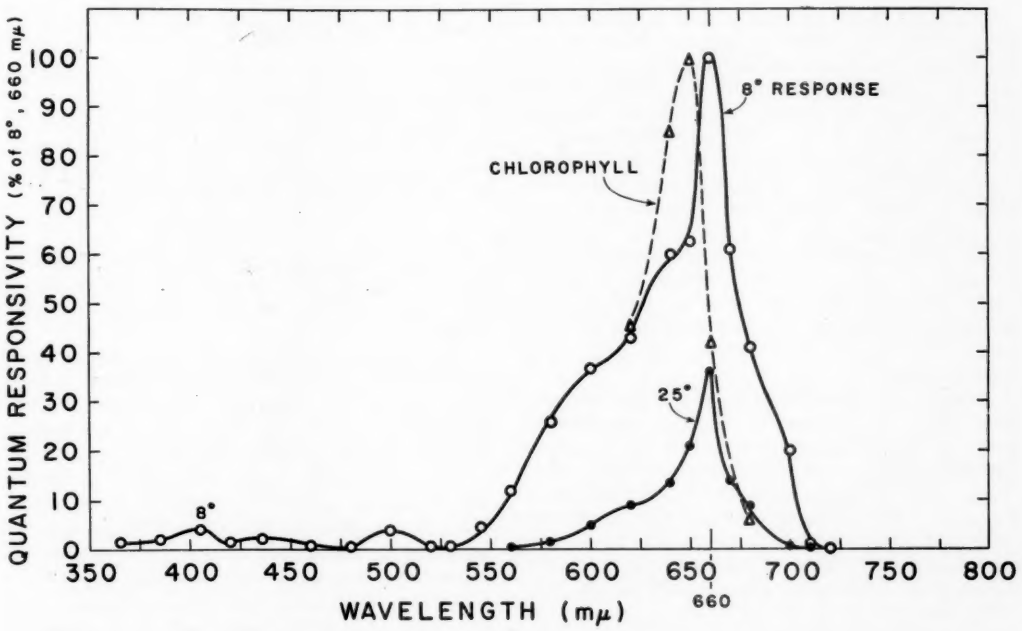


Fig. 3. Action spectrum for photomorphogenic induction. The ordinant is relative quantum responsivity calculated for an 8° or 25° response per unit incident quanta. The ordinant values multiplied by the factor 0.17 convert relative responsivity to absolute responsivity in degrees per nano-einstein/cm². The factor 2.8×10^{-16} converts to degrees per quanta/cm². Dashed curve is for the relative chlorophyll concentration obtained in the hook tissue by a 2.78-hour exposure.

b. Analytical or graphical determination of the energy, E, required to produce a specified response angle, θ':

$$(2) \quad E = \text{antilog } (\theta' - b)/m$$

c. Conversion of energy units from E (mj/cm²) to n (nano-einstein/cm²):

$$(3) \quad n = \lambda E/Nhc = \lambda E/120 = 8.33 \times 10^{-3}\ \lambda E$$

where, λ is wavelength, mμ; N is Avogadro's number, 6.025×10^{14} molecules/nano-mole; h is Planck's constant, 6.625×10^{-31} mj · sec; and c is the speed of light, 3.00×10^{17} mμ/sec.

d. Introducing n into the regression line formula, (1) becomes,

$$(4) \quad \theta = m(\log\ n + \log\ 120/\lambda) + b$$

Thus, the quantum energy curves plotted on a logarithm scale have the same slope as the energy curves of figure 2, but different intercepts.

e. Calculation of quantum responsibility, R, degrees/(nano-einstein/cm²), for hook angles in degrees, θ', of 8° or 25°.

$$(5) \quad R = \theta'/n' = 120\ \theta'/\lambda E'$$

When the response is proportional to the logarithm of the stimulant, various criteria can be set up for the responsivity of the organ. The responsivity can be expressed as the slope of the log energy-response curve (m), as the absolute value of the intercept parameter (b), as a threshold value which would involve the intercept on the energy rather than the angular response axis (– b/m), or as the ratio of response to incident energy when evaluated at a specified response level (R). The last form of expression is used here in calculating the action spectrum curves of figures 3 and 6. However, certain of the other modes of expression are useful in clarifying the probability of existence of minor peaks and other details of the spectrum. Therefore, the slope, m, is plotted in figure 8.

When it was found that the induction action spectrum had a strong peak at 660 mμ, the question arose as to whether the photoactivation of protochlorophyll and its conversion to chlorophyll a might be involved. To test this possibility, the position of the peak of the action spectrum of chlorophyll synthesis was determined by treating hooks in the same manner as for the photomorphogenic induction action spectrum and immediately extracting in acetone. The hooks were irradiated for 2.78 hours, and then, instead of allowing a development period in the dark, the tissue was macerated and assayed for chlorophyll a as described previously (12). The relative concentration of chlorophyll a formed in the hook at several wavelengths in the red are plotted in figure 3 as the dashed curve. The protochlorophyll conversion peak occurred at 650 mμ in accordance with data published by Frank (8) and by Koski et al (14).

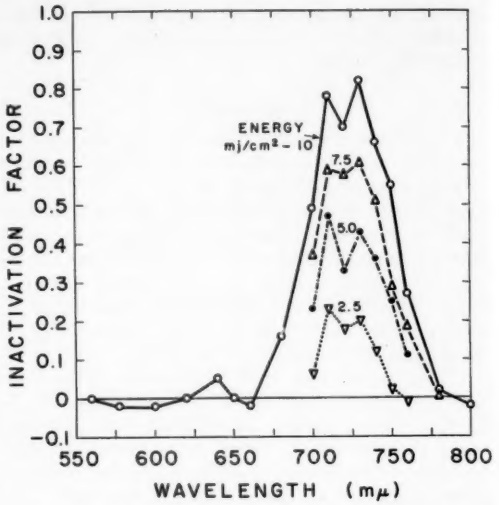


FIG. 4. Equal energy curves for photoinactivation. Data are plotted as inactivation factor vs wavelength at four values of incident energy applied over a 30-min period. Hooks were induced with a red (600 to 700 mμ) exposure of 250 mj/cm² (300 μw/cm² for 14 min) and then inactivated with the energies and wavelengths shown. A factor of 1.0 indicates 100 % reversal; negative values indicate that the reversal treatment increased the hook opening.

PHOTOINACTIVATION: The inactivation spectrum was calculated in essentially the same manner as the induction action spectrum. The inactivation factor, B, was calculated by the following formula:

$$(6) \quad B = (\theta_1 - \theta)/\theta_1$$

where, θ_1 is the hook angle with the induction treatment of 250 mj/cm² applied alone for 14 minutes (50° angle) and θ is the angle obtained after inactivation.

A factor of 1.0 indicates 100 % inactivation ($\theta = 0$) while a factor of zero implies that no inactivation occurred and $\theta_1 = \theta$. A negative value is obtained when the second irradiation increases rather than decreases the effect of the induction treatment.

The inactivation factor obtained from formula (6) is plotted against wavelength in figure 4 for a series of energies at 0, 2.5, 5.0 and 10 mj/cm². The regression curves for each wavelength are presented in figure 5 for the range of 700 to 760 mμ. Up to about 85 % inactivation, the response is proportional to energy. The energy scales have been shifted two units for each curve in order to prevent superposition. The quantum inactivation, β, in per nano-einstein/cm² for 50 % inactivation was obtained from the formula:

$$(7) \quad \beta = 120\ B''/\lambda E''$$

where E″ is the inactivation energy required to give a factor B″ of 0.5.

The quantum inactivation curve is graphed as relative values in figure 6. By multiplying by the factor 1.3×10^{-4}, it is possible to convert to absolute units of inactivation, per nano-einstein/cm^2.

Discussion

Induction: The family of curves for wavelength vs hook angle, as plotted on an equal energy basis in figure 1, shows that the induction response has certain spectral features which consistently appear at all energy levels. The peak at 660 mμ is the most prominent feature and shoulders occur at slightly shorter and longer wavelengths. There is one shoulder in the vicinity of 560 mμ and a second around 620. The long-wavelength shoulder is in the general region of 680 to 700 mμ. The shoulders are most prominently indicated when the slopes of the energy-angle curves are plotted (fig 8). There are two minor peaks in the shorter wavelengths, one between 500 and 520 mμ, and one in the vicinity of 400. All of these features appear in the calculated action spectrum curves of figure 3. Using broad band filters, Weintraub and Price (21) obtained a shoulder in the vicinity of 580 mμ for Avena mesocotyl. The action spectrum for fern spore germination secured by Mohr (16) also shows a shoulder in the vicinity of 600 mμ. It is concluded that this shoulder is evidence of a second minor peak in the pigment contributing to the induction.

The chlorophyll synthesis curve of figure 3 strongly supports the conclusion that protochlorophyll is not directly involved in the photoactivation of the induction mechanism. Since chlorophyll concentration was measured in the hook tissue with the same irradiances and times used for obtaining the induction curves, the two peaks would be expected to coincide if the two processes were being initiated by the same pigment. Further evidence for this conclusion is obtained from data on the effect of far-red energy, beyond the limit for chlorophyll synthesis, on the induction of photomorphogenic responses in the bean seedling (25). It was found possible, by using high energies in the far-red beyond 700 mμ, to induce a significant photomorphogenic reaction without any detectable synthesis of chlorophyll.

While the action spectrum peak of the induction process is at 660 mμ, the shape of the curve probably is strongly influenced by interaction with the inactivation process. The two reactions must compete and they undoubtedly attain various equilibria in the range of about 620 to at least 720 mμ. Therefore, it is quite possible that the absorption peak of the induction pigment is at a somewhat longer wavelength than 660 mμ, possibly between 660 to 680 mμ.

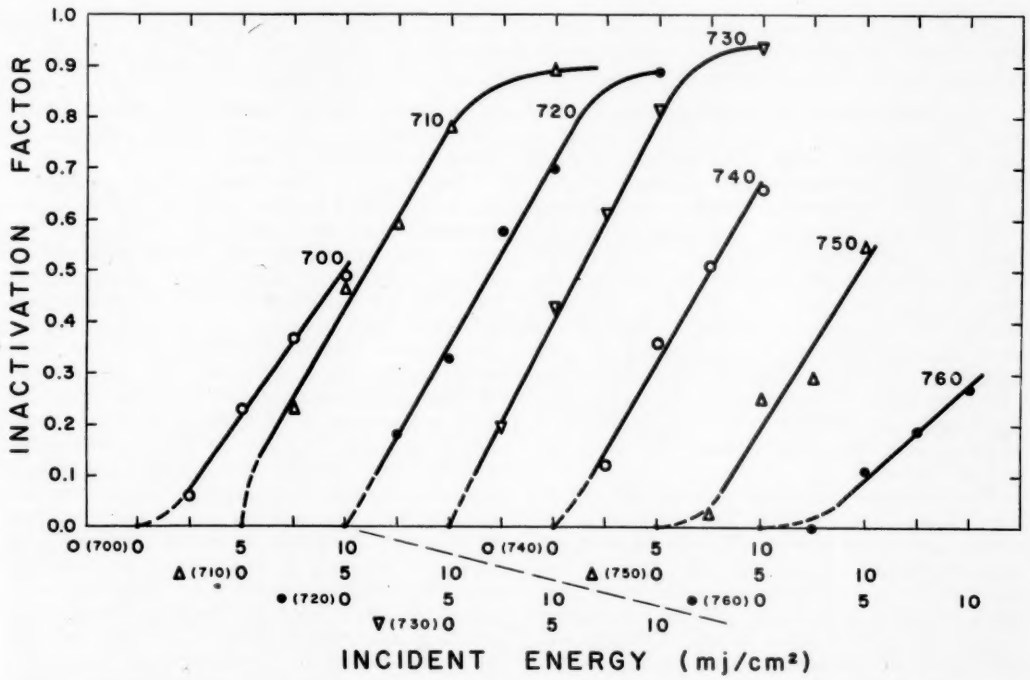


Fig. 5. Photoinactivation regression lines, inactivation factor vs incident energy. Energy scales are shifted two steps to the right for each curve to prevent superposition. Curves for the 710, 720 and 730 mμ wavelengths are extended to 15 mj/cm^2; each curve is extrapolated to zero energy by a dashed line.

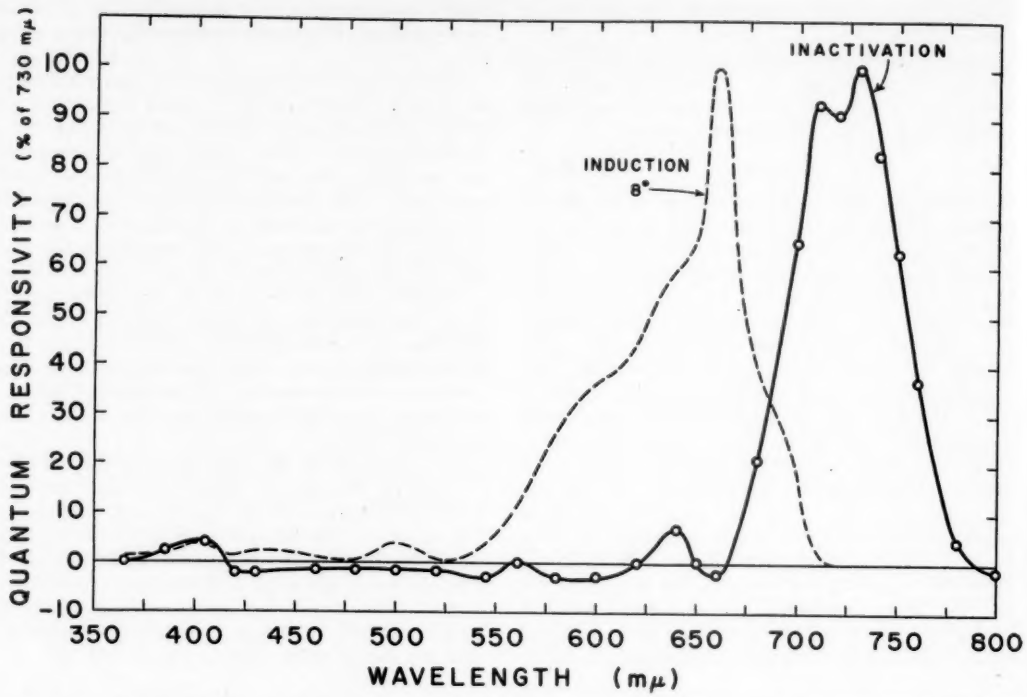
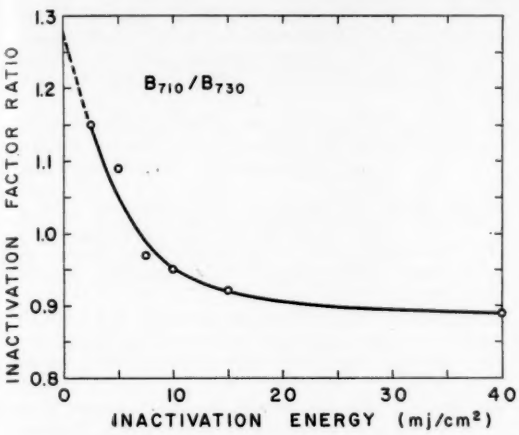


FIG. 6. Photoinactivation (reversal) action spectrum based on a factor of 0.5. Relative quantum responsivity may be converted to absolute values by multiplying the ordinate by 1.3×10^{-4} per nano-einstein/cm^2 or by 2.2×10^{-19} per quantum/cm^2. For comparison, the 8° induction action spectrum is included as the dashed curve.

The non-linearity of the induction process is evident from figure 9, in which the incident energy is plotted against hook angle. The log plots of figure 2 show that the response is closely proportional to the log of the incident energy, especially over the range of 578 to 660 mμ. At shorter and longer wavelengths, the linear nature of the log function is not so consistent. The points at 405, 420, 460 and 560 show evidence of saturation, even when plotted on a log scale. This type of saturation or tendency for the curve to break has been shown for the blue in regard to the development of various organs of the whole seedling (12). The tendency to saturate in the shorter wavelengths may very likely be due to photochemical interactions with the yellow pigments and may be a very indirect effect.

Weintraub and McAlister (20), Weintraub and Price (21) in Avena seedlings, and Parker et al (19) in pea, have shown evidence that the log energy-response curves are straight lines which break at some high value of incident energy and assume a new slope, which is again a straight line. We have no evidence of this type of break, either for the whole bean seedling (12) or for the hook response. Either we have not used sufficiently high intensities in our experiments or the break in an artifact at high intensities. The results of Klein et al (11) have shown that incident energies over a million-fold range fall on the same straight line in regard to hypocotyl straight growth inhibition. The curve breaks only when the shorter wavelengths are used.

INACTIVATION: The equal-energy inactivation curves of figure 4 and the calculated action spectrum of figure 6 show two major peaks at 710 and 730 mμ and a minor one at 640, with a slight peak at about 400 mμ in the blue. When plotted on an equal energy basis, the 710 and 730 peaks shift in relative magnitude with incident energy. At 2.5 and 5.0 mj/cm^2, the 710 peak is dominant, whereas at 7.5 and 10 mj/cm^2, the 730 peak is dominant. The relative values of these peaks were measured and their ratio calculated in the range of 2.5 to 40 mj/cm^2 and the data plotted in figure 7. When this curve is extrapolated to zero energy, the ratio of the 710 and 730 peaks, as evaluated in terms of inactivation factor, extrapolate to about 1.3. This makes it appear that the 710 mμ peak may be the dominant one, and, as the incident energy is increased, it is suppressed by interaction with the induction process which extends into this region. It also is likely that the minor 640 peak may not be a true peak at all, but may result from suppression at 660 mμ by the induction process and that the absorption spectrum of the inactivation pigment actually rises slowly from about 620 mμ to a maximum at 710 mμ. It seems very possible that the absorption spectra of the photoinduction and photore-



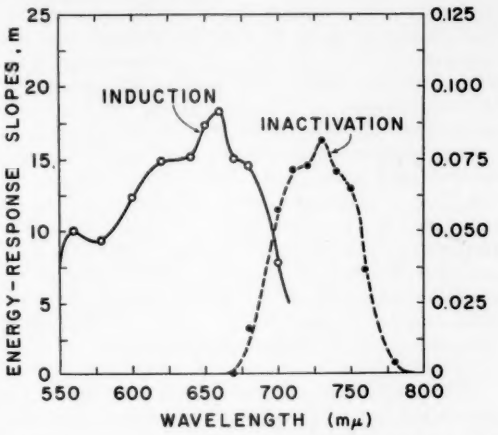


FIG. 7 (*left*). Ratio of the 710 and 730 mμ inactivation factors. The ratio, B_{710}/B_{730}, is plotted against energy.
FIG. 8 (*right*). Regression line-slope values vs wavelength for the induction and inactivation processes. These are the slope constants, m and m′ respectively, derived from the regression line formulas.

versal pigments may be quite similar in shape, with the latter shifted 20 to 30 mμ to the longer red wavelengths.

SUMMARY

The action spectrum of the induction of photomorphogenesis in the bean hypocotyl hook shows certain features which are consistent with the red, far-red groups of reactions in general. The induction response in terms of hook angle vs log of incident energy is linear over the range of at least 0.1 to 30 mj/cm² for the wavelengths of 578 to 660 mμ. At longer and shorter wavelengths, the linearity is not so clear. The action spectrum shows a sharp major peak at 660 mμ, with evidence of two shoulders in the vicinity of 560 and 620 mμ and possibly a third shoulder at 700 mμ. In the shorter wavelengths, two minor peaks appear in the vicinity of 400 and 500 mμ. Because of the close proximity to the peak of inactivation, it is very possible that interaction between the two has shifted the apparent induction peak somewhat toward the shorter wavelengths and that the actual absorption peak of the absorbing pigment is in the range of 660 to 680 mμ.

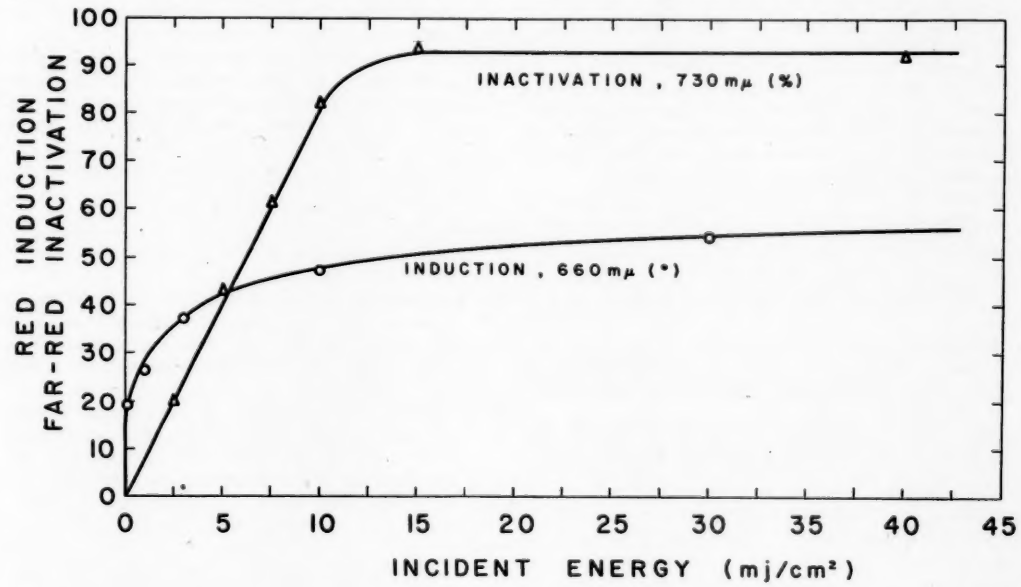


FIG. 9. Energy-response curves for the induction and inactivation processes plotted on a linear energy scale.

The reversal response is a linear function of incident energy up to 85 % reversal. Beyond that, the curve shows sharp saturation at approximately 90 %. The action spectrum for the inactivation process has two major peaks at 710 and 730 mμ, with a minor one at 640. The ratio of the 710 to 730 mμ peaks at various incident energies shows that, at low energies, the 710 peak is dominant and at high energies, the 730 peak is dominant. It is considered very likely that 710 is the principal peak in the absorption spectrum of the photoactivated pigment. The data indicate that the absorption spectra of the pigments responsible for induction and reversal in photomorphogenesis may be similar in shape, with the latter shifted 20 to 30 mμ to the longer red wavelengths.

The authors are indebted to Walter A. Shropshire, Jr. for assistance in calculation of the data and preparation of the figures.

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FACTORS AFFECTING ABSORPTION AND TRANSLOCATION OF FOLIAR APPLIED PHOSPHORUS[1,2]

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Phosphorus is known to be exported from leaves of apple (8, 9, 10), bean (4, 6, 11, 12, 13), chrysanthemum (1, 2), corn (11, 12), tomato (12) and squash (7). The present work with bean plants compares export following application by spray, leaf vein injection (4) and droplet (13) methods. Other factors investigated which affect absorption and subsequent translocation of the spray-applied phosphorus include: 1) wetting agents, 2) phosphorus concentration of the spray, 3) leaf surface (upper vs lower surface), 4) different phosphorus compounds (pH and cation), 5) time, 6) hygroscopic agent, 7) size of area sprayed, 8) age and position of sprayed leaf, and 9) phosphorus level of the plant.

METHODS

Red Kidney bean plants grown in one-half strength Hoagland solution under fluorescent lights on a 6:00 A.M. to 6:00 P.M. day were used throughout. The light intensity, temperature and relative humidity were 1000 ft-c (as measured half-way up the stem), 24° ± 1° C and 60 % ± 4 %, respectively. At least two plants were used for each treatment. To obtain more uniform plants, the axillary buds were removed before vascularization was pronounced, 2 and 3 days before treatment.

The phosphorus solutions containing 0.2 ml of P^{32}-labeled phosphorus of known specific activity were sprayed onto the upper surface of the terminal leaflet of the first trifoliate leaf, unless stated otherwise, between 9:00 and 11:00 A.M. when the plants were 18 days old (12 days after the hypocotyls had straightened). To prevent contamination of the remainder of the plant the leaflet receiving the spray was enclosed in a clear plastic box which was removed several minutes after treatment. Except in the time course of uptake experiments, the plants were harvested and sectioned 24 hours after the application of the tracer. Usually each plant was sectioned into three parts: 1) the sprayed leaflet, 2) the remaining two leaflets and petiole of the first trifoliate leaf, 3) and the remainder of the plant. In some cases the remainder of the plant was divided at the node of the first trifoliate leaf to obtain the upward and downward movement. The amount of phosphorus applied was determined by summation. The percentage of applied phosphorus which moved into the nutrient solution was negligible (about 0.03 %) and was not determined in most experiments.

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[2] This investigation was carried out under U. S. Atomic Energy Commission, Division of Biology and Medicine, Contract No. AT (45-1)-213. Presented at the Seattle meeting of the A.A.A.S., June 12, 1956.

[3] To be submitted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

When autoradiograms were made from quickly dried, pressed whole plants using no-screen x-ray film, the exposure, development and reproduction were uniform so that direct comparisons could be made.

For radioactive assay the various plant sections were wet-ashed in nitric acid, made to volume and the activity determined on aliquots dried on porcelain counting discs using an end-window GM tube. The results were expressed in micrograms of phosphorus or in percentage of the applied phosphorus which was translocated from the sprayed leaflet. The activity applied per plant was between 5 and 20 μc and was constant within each experimental run. P^{32} labeled phosphorus will be referred to as P*.

RESULTS AND DISCUSSION

COMPARISON OF THREE APPLICATION METHODS: Leaf vein injection, droplet, and spray application methods were compared (fig 1) by using a recording rate meter in conjunction with a GM tube (5 mm^2 opening) positioned on the stem 2.5 cm below the node of the treated leaf. The volume of solution used, the number of microcuries of P^{32} employed, and the pH of the solution applied are given in the legend. Figure 2 shows an autoradiograph of treated leaflets (droplet application) from plants represented in tracings 14, 15, and 16. It is obvious that after entry the tracer was carried toward the periphery of the leaflet before entering the phloem for export. The droplet method, to be successful at all, required a pH of approximately 2.0, which produced necrosis at the place of application.

For routine use the spray application proved superior. It gave only a slightly lower concentration of tracer in the stem within a minimum migration period than the injection method, produced no visible injury (except at pH extremes), and it is the method which is best adapted for field use.

EFFECT OF WETTING AGENTS: The following wetting agents were tested at a concentration of 0.05 % of the active ingredient, except where stated otherwise, with spray solutions of 10 mM $NaH_2P^*O_4$: 1) anionic type, Igepon T-77 (sodium *n*-methyl-*n*-oleoyl taurate), Igepon T-78 (ester of sodium isethionate), Tergitol 7 (sodium heptadecyl sulfate), Vatsol OTB (dioctyl ester of sodium sulfosuccinic acid), Vatsol OS (isopropyl naphthalene sodium sulfonate) and 0.5% Dreft (sodium dodecyl sulfate); 2) cationic types; Vatsol C-61 (ethanolated alkyl guanidine amino complex); 3) nonionic types; Vatsol NI (nonyl phenol + 9 moles ethylene oxide) and 0.5 % Carbowax 4000 (polyethylene glycol). None increased and only two, Tergitol 7 and Vatsol OTB, significantly decreased the percentage of phosphorus translocated from the sprayed leaflet in the 24-hour interval.

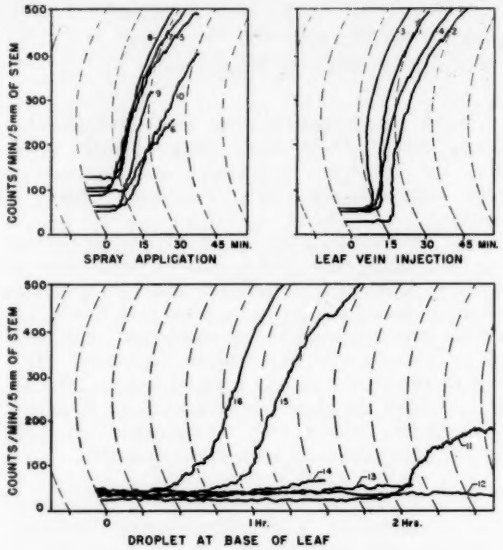

FIG. 1. Continuous rate meter recordings of P^{32} radioactivity in the stems of bean plants 2.5 cm below the node of the treated leaf. Treatment was by three methods described in the text. The volume, P^{32} radioactivity, and the pH of the solutions (adjusted with NaOH) for each curve are as follows: (1) 25 μl, 92 μc, pH 4; (2) 50 μl, 121 μc, pH 4; (3) 25 μl, 167 μc, pH 5; (4) 25 μl, 100 μc, pH 6; (5) 100 μl, 318 μc, pH 4; (6) 100 μl, 318 μc, pH 4; (7) 100 μl, 288 μc, pH 4; (8) 100 μl, 450 μc, pH 4; (9) 100 μl, 336 μc, pH 4; (10) 100 μl, 336 μc, pH 4; (11) 20 μl, 3 μc, pH 1.5; (12) 10 μl, 48 μc, pH 5; (13) 10 μl, 48 μc, pH 0.3; (14) 10 μl, 46 μc, pH 3; (15) 10 μl, 42 μc, pH 2.5; (16) 10 μl, 46 μc, pH 2.

These two latter wetting agents caused the spray solutions to spread very uniformly on the leaf surface and consequently to dry rapidly; three times faster than the controls or the wetting agents of poor spreadability.

Swanson and Whitney (13) found a 90 % reduction in the translocation of P^{32} from a foliar application when Tween 80 (polyoxyethylene sorbitan monooleate) was applied with the tracer. But Fisher and Walker (10) obtained a 7-fold increase in the apparent absorption of phosphorus by McIntosh apple leaves with the addition of Triton X-100 to the KH_2PO_4 spray solution. Apparently some wetting agents form a complex with some phosphate compounds.

Perhaps by using a wetting agent producing a uniform spread a higher concentration of phosphorus could be applied before injury from excessive phosphate occurred, but in many cases this would be more than offset by the smaller volume that could be retained on the leaf surface, by complex formation and by the rapidity of drying.

EFFECT OF PHOSPHORUS CONCENTRATION OF SPRAY: Solutions of 0.2 ml of $NaH_2P^*O_4$ were applied at five different concentrations ranging from 0.3 to 30 mM. No leaf injury was noted. The amount of phosphorus translocated from the sprayed leaflet increased as the amount of applied phosphorus increased, but not in direct proportion (fig 3). The percentage of phosphorus translocated from the sprayed leaflet increased as the concentration of applied phosphorus increased from 0.3 to 10 mM, but no further increase was noted. The decrease with the 30 mM solution was within experimental error. The decrease in the percentage of phosphorus translocated from the treated leaflet at the low phosphorus concentration could be due either to a low rate of absorption or to a high percentage of retention of the applied phosphorus by the leaf tissues, or both.

Whether the 10 mM $NaH_2P^*O_4$ spray was applied to the upper or lower leaflet surface made no significant difference in the percentage of applied phosphorus translocated from the leaflet in the 24-hour interval in spite of the difference in epidermal characteristics of the two surfaces, e.g., number of stomata, thickness and composition of the cuticle and pubescence. This is in agreement with the work of Bennett and Thomas (3) but opposed to Oliver (11).

EFFECT OF DIFFERENT PHOSPHORUS COMPOUNDS (pH AND CATION): The effects of pH and cation on the absorption and translocation of phosphorus from the sprayed leaflet were determined at a concentration of 10 mM phosphorus. $H_3P^*O_4$ and the following three series of phosphorus compounds were used: 1) $NaH_2P^*O_4 \cdot H_2O$, $Na_2HP^*O_4 \cdot 7\,H_2O$, $Na_3P^*O_4$; 2) $KH_2P^*O_4$, $K_2HP^*O_4$, $K_3P^*O_4$; and 3) $NH_4H_2P^*O_4$, $(NH_4)_2HP^*O_4$. The results with the sodium and potassium compounds are shown in figure 4. The 10 mM $H_3P^*O_4$ solution injured the sprayed leaflet and only 4.7 % of the applied phosphorus was exported. About 6.0 % of the phosphorus was exported with both ammonium compounds. A second sodium

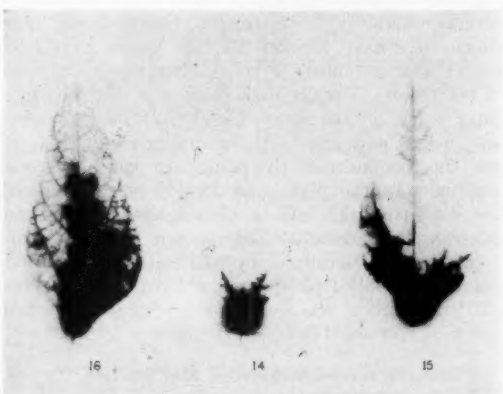

FIG. 2. An autoradiogram of the treated leaflets of the plants represented in tracings 14, 15 and 16 of figure 1. The leaflets were severed from the plants at the times indicated by the termination of their respective tracings.

series, in duplicate, at 0.3 mM phosphorus gave the following average translocation percentages: $H_3P^*O_4$, 6.0; $NaH_2P^*O_4$, 2.6; $Na_2HP^*O_4$, 0.6; $Na_3P^*O_4$, 2.1. No injury from $H_3P^*O_4$ was evident at this lower concentration.

It seems unlikely that the rather large variability in individual plants can be attributed to chance differences in the distribution of the spray from the same sprayer. It is more likely that plants differ individually and from time to time in their ability to translocate phosphorus.

The results with 0.3 mM phosphorus are similar to those obtained by Swanson and Whitney (13). They attributed the increased effectiveness of absorption and translocation at the lower pH to a suppression of the dissociation of phosphoric acid, and to a possible direct effect on the permeability of the adjacent cells. But they worked only with sodium phosphates, and when the potassium curve of figure 4 is taken into consideration it is evident that their explanation is not tenable. The translocation of phosphorus from the $H_2PO_4^-$ form of the sodium and potassium compounds exhibited striking differences. $KH_2P^*O_4$ yielded the lowest and $NaH_2P^*O_4$ the highest translocation in their respective series. The differences in absorption and translocation cannot be explained as being due to pH since the cation had considerable effect.

It was observed that NaH_2PO_4 and K_2HPO_4, the two compounds from which translocation was highest, did not crystallize on the leaflet as rapidly as KH_2PO_4, Na_2HPO_4, Na_3PO_4, $NH_4H_2PO_4$ and $(NH_4)_2HPO_4$. The assumption was made that their effectiveness in supplying phosphorus was due to their effective retention of moisture. Two experiments were performed to test this assumption. The moisture retention of the sodium and potassium compounds was determined as follows. Known duplicate quantities of each of the compounds were dissolved in a small amount of water in individual containers and then reweighed after drying 24 hours. The values obtained were plotted in figure 5, together with the average values for the percentage of phosphorus translocated from the previous experiment. The amount of phosphorus translocated parallels the moisture retention in all cases with the exception of K_3PO_4. K_3PO_4 had the greatest moisture retention but failed to support a corresponding efficient translocation, probably because of its high pH.

In order to test the effects of a hygroscopic agent, glycerin, ethylene glycol and propylene glycol were added to ten 1 mM solutions of $KH_2P^*O_4$ and $K_2HP^*O_4$ which, in turn, were used in translocation studies as above. The results are shown in figure 6. The moisture retention was determined in the same manner as before, except that one drop of the additives was included after the initial weighings. Moisture retention was greatly increased with glycerin but essentially unaltered with the glycols. Glycerin raised the percentage of phosphorus translocated from $KH_2P^*O_4$ solutions from 2 to 14 but reduced the percentage translocated from $K_2HP^*O_4$ solutions from 10 to 4. The glycols did not increase translocation significantly and were not hygroscopic.

The results with glycerin and $KH_2P^*O_4$ are in agreement with the results of Fisher and Walker (10) who observed that absorption of $KH_2P^*O_4$ by apple leaves was more than doubled with the addition of glycerin and more than tripled by rewetting the sprayed areas 3 times in the 24-hour period.

The decrease in phosphorus translocation when glycerin was included in the $K_2HP^*O_4$ spray solution might be accounted for by the formation of a $K_2HP^*O_4$-glycerin complex. Evidence for this was obtained by chromatographic methods which indicated that a K_2HPO_4-glycerin complex, but not a KH_2PO_4-glycerin complex, may be formed. Any complex would, of course, decrease the effective phosphorus concentration and thereby reduce phosphorus translocation. Silberstein and Wittwer (12) found growth to be less from the use of foliar applied potassium glycerophosphate than from H_3PO_4, KH_2PO_4 or $NH_4H_2PO_4$ solutions. Support is thereby given to the idea that the hygroscopicity of a foliar applied fertilizer is of prime importance, but complex formation is to be avoided.

Time Course of Uptake: The course of uptake of phosphorus over a period of 96 hours was determined with equal quantities of 10 mM $NaH_2P^*O_4$. Figure 7 shows both the percentage of phosphorus absorbed and translocated from the sprayed leaflet, and that retained in the sprayed leaflet. To obtain these values the unabsorbed phosphorus was washed from the sprayed leaflet at harvest time by a low-pressure jet of dilute HNO_3 containing a wetting agent (Tergitol 7) (10). The other essential value, the total amount of phosphorus actually applied, was determined by summation.

It is evident that after 30 hours, when about one-half of the applied phosphorus had been absorbed, uptake fell off markedly. The phosphorus translocated from the leaflet continued to increase thereby actually reducing the amount retained within the leaflet. By the end of the 96-hour period 60 % of the applied phosphorus had been absorbed and more than half of this had been translocated away from the leaflet. Using the corresponding potassium salt at 14.7 mM, Fisher and Walker (10) found a 30 % absorption in 4 days by apple leaves. Absorption appears to continue for some time after application (8, 9, 10, 11). The break in the absorption curve probably was due to drying of the spray to the point of crystallization. Rewetting of the sprayed area, raising of the relative humidity, or the presence of a hygroscopic agent might be expected to delay this break; the use of the corresponding potassium salt might hasten it.

In order to compare the amount of applied material moving upward and downward within the plants, they were divided at the node of the first trifoliate leaf at harvest time. It was found that within the first half hour more than 6 times more phosphorus

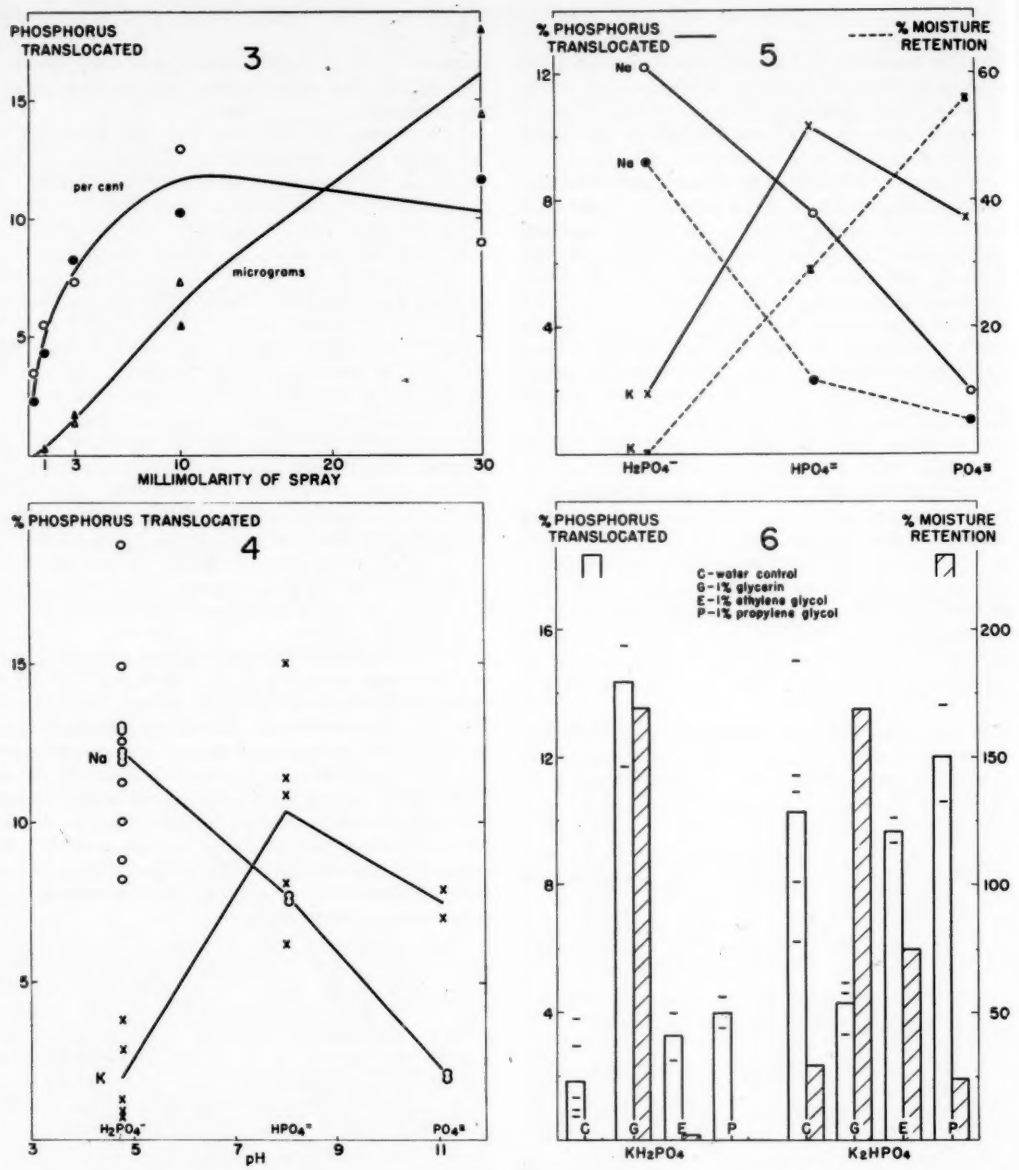

FIG. 3. Percentage and micrograms of phosphorus translocated from the terminal leaflet of the first trifoliate leaf in 24 hours by the spray application of 0.2 ml of five different concentrations of $NaH_2P^*O_4$. Open circles and triangles, expt 1; solid circles and triangles, expt 2.

FIG. 4. Percentage of applied phosphorus translocated from the terminal leaflet of the first trifoliate leaf in 24 hours by the spray application of 0.2 ml of a complete series of sodium (O) and potassium (X) phosphates. The pH's and anions of the spray solutions are shown on the abscissa.

FIG. 5. Relation of the percentage of phosphorus translocated (average values from figure 2) from a leaflet sprayed with 0.2 ml of 10 mM $NaH_2P^*O_4$, $Na_2HP^*O_4$, $Na_3P^*O_4$, $KH_2P^*O_4$, $K_2HP^*O_4$, or $K_3P^*O_4$ solutions to the percentage of moisture retention of similar solutions.

FIG. 6. Relation of the percentage of phosphorus translocated in 24 hours from a leaflet sprayed with 0.2 ml of 10 mM $KH_2P^*O_4$ or $K_2HP^*O_4$ solutions containing no additive, 1 % glycerin, 1 % ethylene glycol, or 1 % propylene glycol to the percentage of moisture retention of similar solutions. The short horizontal lines represent experimental values, and the vertical bars represent averages.

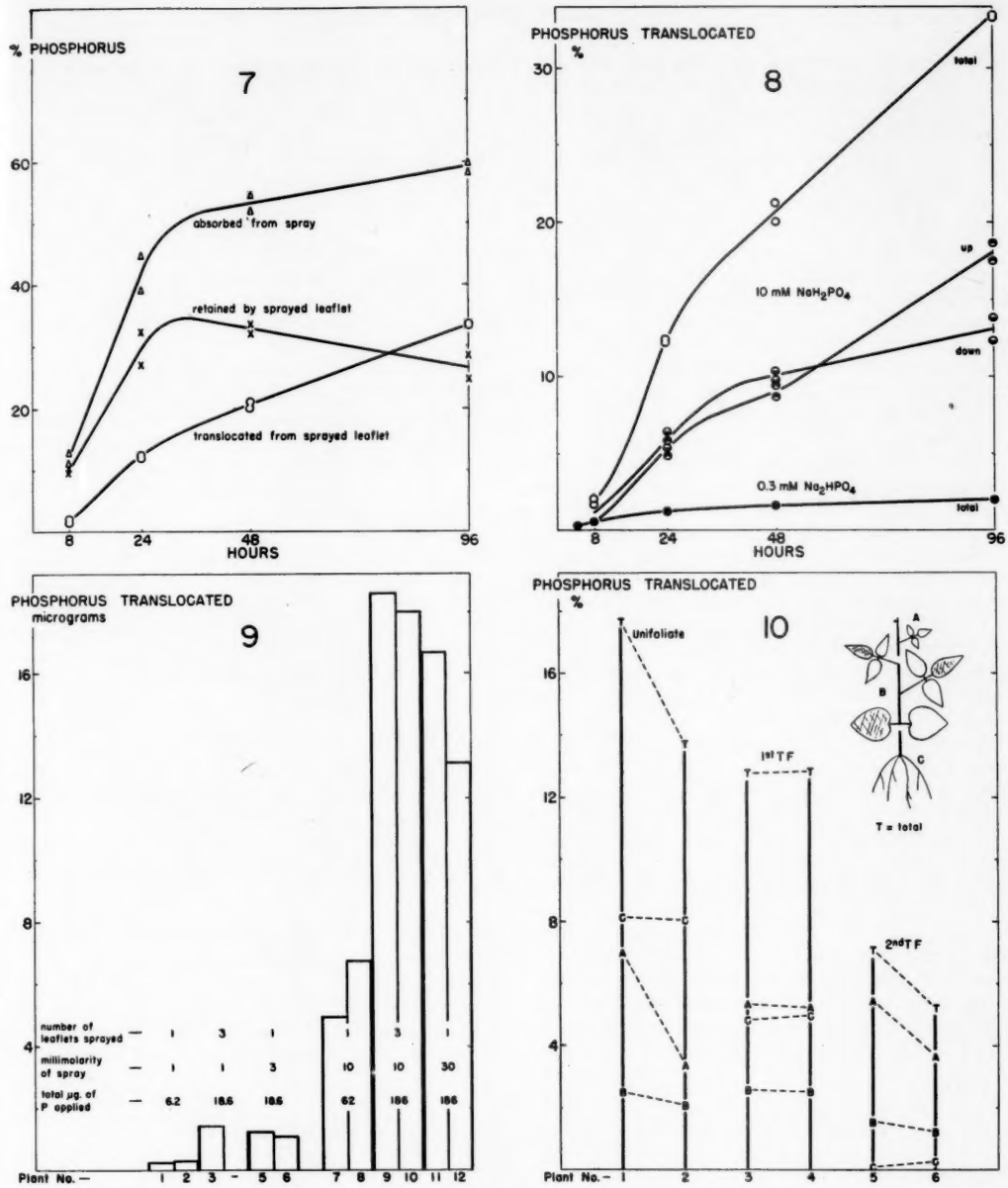

FIG. 7. Percentage of phosphorus—absorbed from the spray solution, retained by the sprayed leaflet, and translocated from the sprayed leaflet—at various intervals after treatment with 0.2 ml of 10 mM $NaH_2P^*O_4$.

FIG. 8. Percentage of applied phosphorus translocated from the sprayed leaflet into the remainder of the plant (total), the portion of the plant above the first trifoliolate node (up), and the portion below this node (down) at various intervals after treatment with 0.2 ml of either 10 mM $NaH_2P^*O_4$ or 0.3 mM $Na_2HP^*O_4$.

FIG. 9. The micrograms of phosphorus translocated in 24 hours from the terminal leaflet or from all three leaflets of the first trifoliate leaf when sprayed with 0.2 ml of 1, 3, 10, or 30 mM $NaH_2P^*O_4$. The object was to compare translocation with the area (number of leaflets) treated.

FIG. 10. Percentage of phosphorus translocated from leaves of different positions on the stem into three plant sections, A, B, and C, as indicated in plant diagram. Plants 1 and 2 are duplicates and received 0.2 ml of $NaH_2P^*O_4$ on a unifoliate leaf. The terminal leaflet of the first trifoliate leaf of plants 3 and 4, and the second trifoliate leaf of plants 5 and 6 received similar amounts of tracer.

from the spray was found below this node than above it. This ratio was decreased to 2.5 at the 8-hour interval, and after 96 hours more phosphorus from the spray was found above this node than below it (fig 8). The same relationship was observed in both experiments, i.e., with 0.3 mM $Na_2HP^*O_4$ and with 10 mM $NaH_2P^*O_4$ where the percentage exported in the 96-hour period was 2.0 % and 34.5 %, respectively.

Effect of the Size of Area Sprayed: To determine the relationship between the translocation of phosphorus and the leaf area treated, the spray was applied to either the terminal leaflet or all three leaflets of the first trifoliate leaf. Concentrations of 1, 3, 10 and 30 mM phosphorus were used in the experimental design selected to show this relationship. The design and the results are shown in figure 9. Each spray treatment involved 0.2 ml of solution.

The lateral leaflets appeared comparable to the terminal leaflet in translocation since three leaflets together translocated about three times as much as one leaflet when using 10 mM phosphorus. Within the concentration range used, the area sprayed seemed to make little difference. The amount of phosphorus applied appeared to be more important than the area of treatment, however the area factor is not without its effect when more concentrated solutions were used since one leaflet sprayed with 30 mM phosphorus exported slightly less phosphorus than three leaflets sprayed with 10 mM phosphorus.

Effect of the Age and Position of the Sprayed Leaf: From figures 10 and 11 it can be seen readily that the age and position of the sprayed leaflet determined the amount and direction of translocation from the treated leaf. The results can be summarized as follows: 1) the amount of phosphorus translocated from a leaf was proportional to its age, and therefore to its position; the older leaves translocated the most phosphorus while the young immature leaves high on the stem failed to export phosphorus (Because of its size only 0.1 ml instead of the usual 0.2 ml of spray was used on the small, third trifoliate leaflet.); 2) the amount of phosphorus translocated from a leaf to the root was proportional to the proximity of the leaf to the root; 3) all leaves exporting phosphorus, regardless of age or position, contributed approximately equal amounts of phosphorus to the immature leaves at the stem apex. It should be stressed that these were young vigorous plants, and there was no sign of senescence of the primary leaves.

Most of the variability between duplicate plants in the percentage of applied phosphorus translocated can be attributed to section A, the stem tip where rapid growth was occurring. This is in agreement with work of Biddulph (5). Section A of plants 1 and 5 had lower dry weights than plants 2 and 6.

Steaming the petiole of the sprayed leaf completely blocked the passage of the P^{32} from the leaf. The xylem was still functioning as the leaf did not wilt during the 24-hour interval. The phloem, then, was the tissue involved in the exportation of the phosphorus as S. F. Biddulph (6) has shown by means of microautoradiograms.

Effect of the Phosphorus Level of the Plants: In all previous experiments the plants were grown in nutrient solutions with a 0.5 mM phosphorus level. If the level was reduced to 0.05 mM, there was no appreciable change in the amount of applied phosphorus translocated from the sprayed leaflet in 24 hours, and the foliage was comparable in appearance to the plants grown at the higher level, but the roots were slightly darker. However, plants grown without phosphorus in the nutrient solution were darker green in color, much smaller and translocated only one-fifth as much of the applied $NaH_2P^*O_4$ as plants grown at the higher phosphorus levels. From this it is apparent that phosphorus translocation is impaired in phosphorus deficient plants.

Effect of Daily Spraying of Foliage on the Phosphorus Concentration of Roots: Four plants were grown at each of three phosphorus levels, 0.50, 0.05 and 0.00 mM. Two plants at each level were sprayed daily for 10 days, from the 9th to the 18th day, with unlabeled 30 mM NaH_2PO_4. Both the upper and lower surfaces of every leaf were sprayed

Table I

Increase of Root Phosphorus from Daily Spraying of Foliage of Plants Grown at Three Phosphorus Levels

P conc of nutrient soln, mM	Sample	Dry wt roots (mg)		P in roots (mg)		Diff.	% Increase of root P	% of spray P in root *
		Sprayed	Unsprayed	Sprayed	Unsprayed			
0.50	1	307	326	6.15	6.4			
	2	396	341	7.3	6.35	...	...	...
	Av.	352	334	6.7	6.4			
0.05	1	427	415	2.6	1.8			
	2	401	353	2.7	1.5			
	Av.	413	384	2.65	1.65	1.0	61	14
0.00	1	361	391	1.4	0.78			
	2	313	305	1.3	0.61			
	Av.	337	348	1.35	0.70	0.65	94	9.1

* Total of 7.2 mg of phosphorus applied per plant.

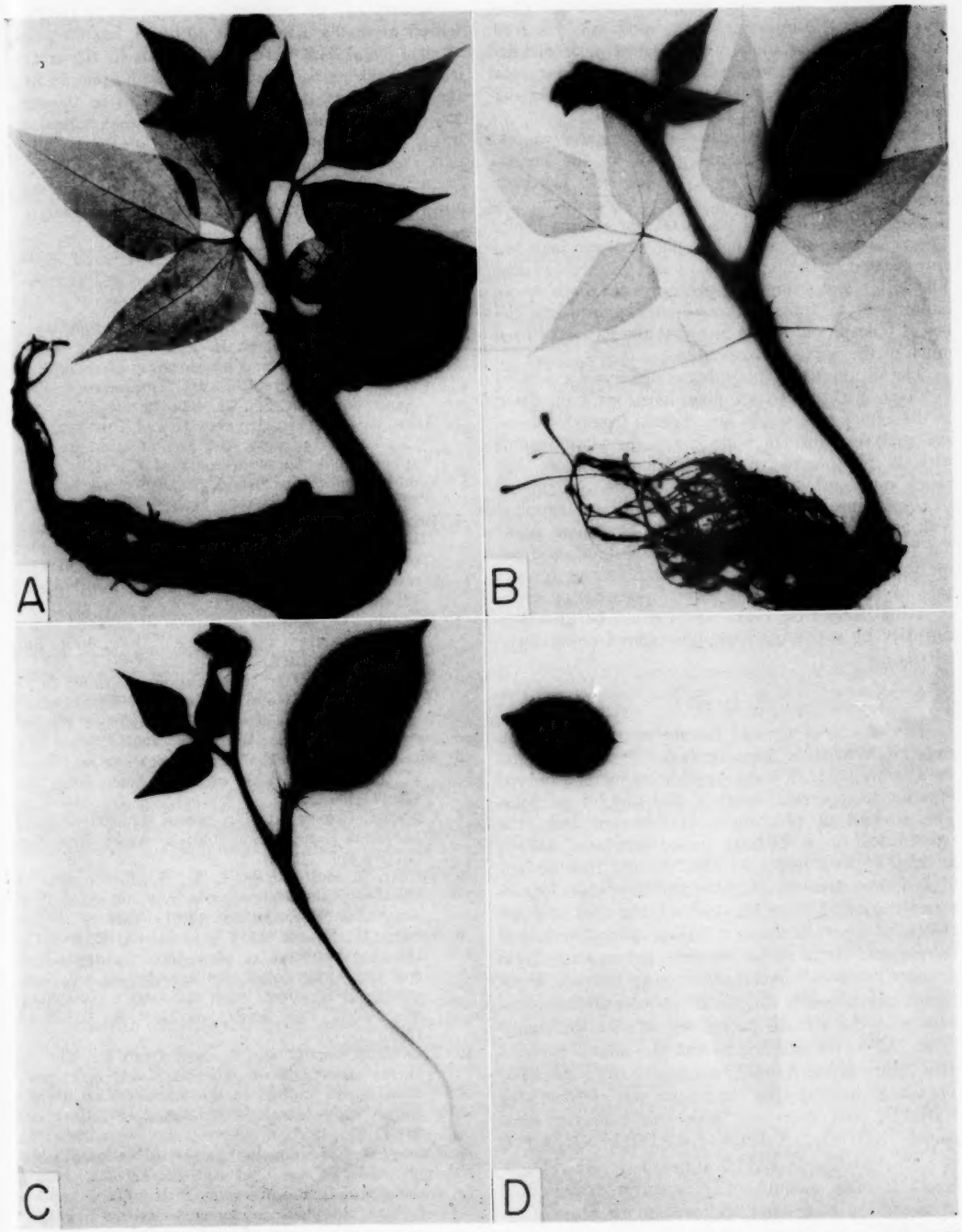

FIG. 11. Autoradiograms demonstrating the distribution of P^{32} in bean plants 24 hours after the spray application of 0.2 ml of 10 mM $NaH_2P^*O_4$ to the following leaves: (A) a unifoliate leaf, (B) the terminal leaflet of the first trifoliate leaf, (C) the terminal leaflet of the second trifoliate leaf, (D) the terminal leaflet of the third trifoliate leaf. Axillary buds were not removed.

each morning. The total amount applied per plant was 7.2 mg. No injury from the spray was observed. After harvest the roots were washed with distilled water, oven dried, weighed, and analyzed for total phosphorus. The remainder of the plant was discarded. The results are shown in table I.

No significant increase in the phosphorus content of the roots of the plants grown at the normal (0.5 mM) phosphorus level was noted. However, increases of 61 % and 94 % were noted in root phosphorus at the 0.05 mM and 0.00 phosphorus levels. The percentage of the applied phosphorus which was translocated to the roots was 14 and 9.1, respectively. The latter low value was probably due both to an impairment of translocation through phosphorus deficient phloem and to the fact that the foliage utilized much of the applied phosphorus.

The symptoms of phosphorus deficiency exhibited by plants with inadequate phosphorus were alleviated to the extent that would be expected from the chemical analysis shown in table I, i.e., the plants grown without phosphorus in the nutrient solution, but which were sprayed daily with phosphorus, attained an appearance midway between the plants supplied with ample phosphorus and the plants without phosphorus either from the nutrient solution or from sprays. They were rapidly approaching the appearance of the plants at the normal phosphorus levels. It seems likely that bean plants could be grown to maturity by supplying their phosphorus requirement foliarly.

Summary

The effects of various factors on the absorption and translocation of foliar-applied P^{32} in bean plants were determined. A spray application method proved superior to leaf vein injection and droplet methods. The amount of phosphorus translocated from the treated leaf in a 24-hour period increased as the amount applied increased; and it was independent of leaf area treated, except possibly at the highest concentration (30 mM), and of the leaf surface treated (upper or lower). More phosphorus was translocated from older (lower) leaves than from younger (upper) leaves (very young leaves did not export phosphorus), and the leaves contributed phosphorus to the root in proportion to their proximity to it. All leaves which exported phosphorus contributed approximately equal amounts to the stem apex. Translocation of applied phosphorus was greatest with NaH_2PO_4 and decreased with the following compounds: $K_2HPO_4 > K_3PO_4 \cong Na_2HPO_4 \cong NH_4H_2PO_4 \cong (NH_4)_2HPO_4 > H_3PO_4$ (injury) $> KH_2PO_4 \cong Na_3PO_4$. The amount of phosphorus translocated from a given compound appeared to be directly related to the drying time of the solution on the leaf. Glycerin increased the translocation from KH_2PO_4 to the level of NaH_2PO_4 but reduced the translocation from K_2HPO_4, probably because a complex was formed.

Absorption of applied phosphorus (NaH_2PO_4) fell off markedly after about 30 hours, but 60 % was absorbed and 34.5 % translocated out of the treated leaflet in 96 hours. The amount of phosphorus from the treated leaf that moved downward in the stem was initially greater than the amount that moved upward, but after 48 hours accumulation in the upper part exceeded than in the lower.

Surface active agents (nonionic, anionic and cationic) were ineffective in increasing translocation.

The authors wish to express their gratitude to Mr. Robert Cory for his technical advice.

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THE PRESENCE OF ORNITHINE CYCLE AMINO ACIDS IN SOME HIGHER PLANTS[1,2]

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Although there have been many investigations on the presence of free amino acids in higher plants, there are few reporting the simultaneous occurrence of the ornithine cycle amino acids, ornithine, citrulline, and arginine. Few deliberate attempts have been made to demonstrate the presence of the ornithine cycle amino acids in a single higher plant. Work from Virtanen's laboratory showed that these amino acids were present in the alder (11) but not in the pea (10). Wheat leaves (12), barley leaves (14) and wheat roots (6) have been shown to contain free arginine but these plant materials were not critically investigated for the occurrence of free citrulline or ornithine. Although citrulline was first discovered in the watermelon fruit (18) it was not known whether this or the other amino acids were present in seedlings of this plant. This paper reports studies on the occurrence of ornithine cycle amino acids in some higher plants, as a first step in a study of the ornithine cycle.

MATERIALS AND METHODS

Wheat (var. Big Club 43), barley (var. Sacramento) and watermelon (var. Klondike) seeds were stored at room temperature and treated with the fungicide "Semesan" prior to germination. Seeds were soaked 24 or 48 hours in aerated tap water, germinated between two layers of dampened cheesecloth and grown in the dark at room temperature. After 8 to 10 days the wheat and barley plants were grown in Hoagland's complete nutrient solution in the light of an east window. These plants were harvested up to the 3-leaf stage or when they were 10 to 21 days of age. The leaves and roots were removed, rinsed and frozen or extracted immediately. The watermelon seedlings were maintained in the dark on tap water and were usually harvested after 18 to 20 days when they were 13 to 15 cm in length. The watermelon sprouts and roots were extracted together.

Variable quantities of plant material were cut into ten times their weight of boiling 70 % ethanol. The solution was boiled 5 minutes and blended in a Waring blendor for 5 to 10 minutes. The suspension was usually allowed to stand overnight at room temperature when the liquid was decanted and the residue re-extracted with 70 % ethanol. The residue, after separation by filtering through cheesecloth or centrifugation, was washed with small portions of ethanol. The combined solutions were evaporated to dryness on the steam bath. The residue was extracted with water 4 or 5 times and the washings were combined and made up to volume with water.

Paper chromatograms were run by the method of Levy and Chung (9) or by a descending technique on large sheets of Whatman No. 1 paper with the following solvents: *N*-butanol–acetic acid–water (52 : 12 : 25 or 4 : 1 : 5) (BAW), water-saturated phenol (phenol), 95 % ethanol–ammonium hydroxide (95 : 5) (ethanol-NH_3) and methanol–triethanolamine–water (80 : 4 : 16) (MTW). The R_F values used to identify amino acids on chromatograms of plant extracts were compared only after a suitable correction was applied with asparagine as an internal standard. The amino acid spots usually were made visible by dipping the chromatograms in 0.5 % ninhydrin in acetone (16) and drying them for 15 minutes at 60° to 80° C.

The ornithine cycle amino acids were identified by the corrected R_F values in a number of solvents and treatment of the chromatograms with semispecific sprays and dips. *p*-Dimethylaminobenzaldehyde (PDB) in HCl (4) gave a yellow color with citrulline and other ureido compounds such as allantoin, allantoic acid and urea. Some typical R_F values for the compounds are shown in table I.

TABLE I

R_F VALUES OF UREIDO COMPOUNDS IN VARIOUS SOLVENTS

SOLVENT	COMPOUND			
	CITRULLINE	ALLANTOIN	ALLANTOIC ACID	UREA
Cresol (9)	0.41	0.37	0.07	...
BAW	0.15	0.18	0.18	0.34
Phenol	0.62	...	...	0.69

Citrulline was readily distinguished from allantoic acid and urea in the cresol and BAW solvents, respectively. Allantoin and citrulline could only be differentiated on a chromatogram by their R_F values with use of the internal standard technique. Ornithine was distinguished from other amino acids by R_F values and treatment of chromatograms with the specific vanillin reagent (3). Suitable spots were obtained on chromatograms with 2.5 μgm and 5.0 μgm of ornithine run in BAW and MTW solvents, respectively.

A semispecific spray (1) based on a modified Sakaguchi reaction was used for arginine, but addition of KOH (or K_2CO_3) to the *a*-naphthol solution was not necessary. A distinct spot appeared with as little as 1.5 μgm arginine after chromatography in BAW. Guanidinoacetic acid (glycocyamine) gave a positive reaction but its R_F value on BAW chromatograms was 0.22 as compared to 0.08 for arginine.

Compounds present in extracts of large quantities of plant material and column eluates were further

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[2] Portion of a thesis submitted by R. Kasting in partial fulfilment of the requirements for the Ph.D. degree at the University of California, Berkeley, September, 1956.

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purified and separated by band paper chromatography. The compounds were identified from adjacent authentic compounds or a strip from each end of the band was removed and treated with a suitable color reagent. The area containing the compound was cut out and the substance was eluted with water.

The amino acids also were separated and concentrated by elution from Dowex-2 (OH form) or Dowex-50 (H-ion form) ion exchange columns with ammonium hydroxide or hydrochloric acid (15). The ammonium hydroxide elution procedure used 100 ml of the following solutions in the order listed: H_2O, 0.01 M, 0.1 M, 1.0 M, 2.0 M, and 4.0 M NH_4OH. The acid elution procedure was most satisfactory because of less interference from other substances in the subsequent paper chromatography.

Total quantities of free amino acids were measured by a quantitative paper chromatographic method (13). Suitable quantities of plant extract were chromatographed on two-dimensional chromatograms in BAW and phenol. The air-dried papers were dipped in ninhydrin solution, dried at room temperature and the amino acid spots were immediately cut out and placed in the KCN, pyridine, phenol solution of Troll and Cannon (17). Similar sized sections of the chromatogram free of amino acids were treated in the same manner for blank values. After the color was developed (17) the solutions were made up to volume and centrifuged to remove shreds of paper. The optical density was measured at 570 $m\mu$ with a Beckman Model-B spectrophotometer. Equimolar concentrations of most amino acids gave color densities similar to arginine (17) which was used as standard.

Ornithine was determined in some samples by the colorimetric method of Chinard (2). In wheat extracts, the quantities of ornithine were estimated from the size and color density of spots as compared to standard solutions after treatment of chromatograms with vanillin.

Citrulline in watermelon extracts was measured by the modified carbamido-diacetyl method (8) and a combination paper chromatographic, densitometric method (19). The photometric method was unsuitable for wheat-leaf extracts because they contained allantoin and urea. The citrulline content of wheat leaves was estimated by visual comparison of the size and color density of spots on chromatograms of extract and standard solutions after treatment with PDB.

Arginine was determined directly in plant extracts or purified samples by the method of Dubnoff (5). Paper chromatographic analyses of extracts showed that other Sakaguchi-reacting substances were of no quantitative significance.

Results

Wheat Roots: Figure 1 shows a chromatogram map of the alcohol-soluble ninhydrin reacting substances found in 33 mg of fresh wheat root. There was no evidence of citrulline or ornithine in this sample.

Citrulline: One- and two-dimensional chromatograms of extracts representing 500 mg of root after

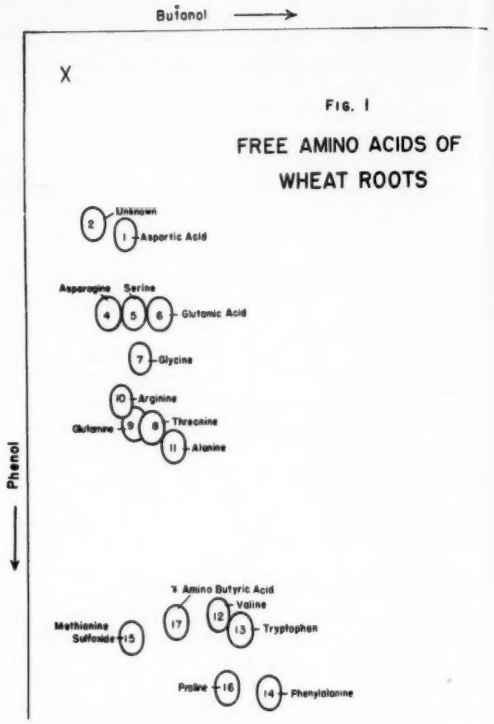

FIG. 1. Two-dimensional chromatogram map showing the amino acids present in alcohol extract representing 33 mg of fresh wheat root.

PDB and ninhydrin treatment showed that if citrulline was present it was in quantities of less than 0.03 micromole per gram fresh weight. Two-dimensional chromatograms in BAW and phenol of solutions representing 2.5 and 3.2 grams of roots which were previously purified by band paper chromatography in BAW or phenol, respectively, showed the presence of a compound which gave the PDB reaction and R_F values of citrulline. Because allantoin was present in the roots it cannot rigorously be excluded as a contaminant. Lower R_F values in the BAW solvent of the unknown spot as compared to allantoin indicate the compound most probably was citrulline.

Ornithine: There were no obvious ornithine spots on well defined, two-dimensional chromatograms in BAW and phenol representing about 50 mg of root. The alcohol extract from 18 grams of fresh roots was given a preliminary purification on a Dowex-50 column with ammonium hydroxide elution. The eluates which normally would have contained ornithine were purified further by band paper chromatography in BAW and MTW. A two-dimensional chromatogram in BAW and MTW of the resulting solution when treated with vanillin gave a spot with the R_F values and distinctive color of ornithine. The ornithine concentration was estimated to be 0.01 micromole per gram fresh weight of roots.

Arginine: Two-dimensional chromatograms with and without the Sakaguchi reagent treatment confirmed (6) the presence of arginine in the alcohol extracts of wheat roots.

BARLEY LEAVES, *Citrulline:* One-dimensional chromatograms of untreated extracts and extracts, purified by band paper chromatography, representing up to 500 mg of fresh leaves showed traces of allantoin but no recognizable citrulline. The results suggest that the upper limit for the citrulline content of the barley leaves was less than 0.03 micromole per gram. Extract equivalent to 3.2 grams of leaves was investigated after a preliminary purification by band chromatography. A two-dimensional chromatogram gave a PDB-positive spot in the citrulline area but it was not possible to decide whether it was caused by allantoin, citrulline or both.

WHEAT LEAVES: Figure 2 is a chromatogram map of the free amino acids and ninhydrin-positive substances found in 100 mg of fresh wheat leaves after chromatography by the method of Levy and Chung (9). There was no evidence for ornithine or citrulline but arginine was present.

Citrulline: Two-dimensional chromatograms of extract representing 200 mg of leaves and PDB treatment showed a trace of citrulline and allantoin or both. The maximal quantity of citrulline in the leaves was less than 0.07 micromole per gram. Extracts representing from 1.5 to 3.2 grams of fresh leaves were purified by band paper chromatography, the resulting solutions were chromatographed, and the papers treated with PDB. Two chromatographic spots suggested the presence of citrulline and allantoin. Subsequent experiments for verification with larger amounts of plant material showed measurable amounts of each. The presence of citrulline in wheat leaves was confirmed by chromatographic identification in leaf extract from 8.8 grams of plant material separated on a Dowex-50 column with ammonium hydroxide elution. This treatment separated allantoin and citrulline, the former was present in the water wash, the latter in the 1M NH_4OH eluate. The quantity of citrulline recovered from the column was about 0.003 micromole per gram fresh leaves, and allantoin 0.01 micromole per gram.

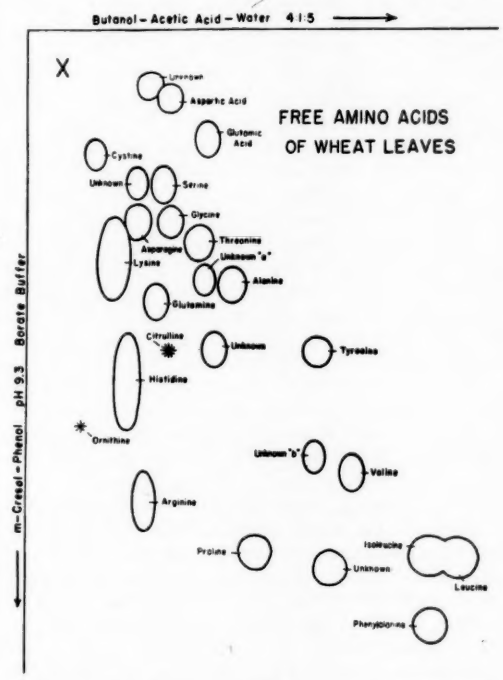

FIG. 2. Two-dimensional chromatogram map of the free amino acids present in 100 mg of fresh wheat leaves.

TABLE II

QUANTITATIVE ESTIMATES OF THE ORNITHINE CYCLE AND COMMON AMINO ACIDS IN WHEAT LEAVES AND WATERMELON SEEDLINGS

AMINO ACID	MICROMOLES/GM FRESH PLANT TISSUE	
	WHEAT LEAVES	WATERMELON SEEDLINGS
Ornithine	0.01	0.2
Citrulline	0.01	0.7
Arginine	0.8	0.9
Aspartic acid	0.8	1.1
Glycine	0.5	0.9
Serine	0.8	1.5
Threonine	0.5	0.5
Alanine	0.7	2.6
Glutamic acid	0.6	0.7
Valine	0.6	0.7
Leucines	0.6	1.0
Tyrosine	0.4	0.1
Asparagine	High	Not detected

Ornithine: The extract from 1.8 grams of leaves was placed on a Dowex-2 column and the water eluate was investigated for ornithine. The concentrated eluate was further processed by band paper chromatography and the resulting sample was chromatographed in two-dimensions in BAW and MTW. Vanillin treatment of the chromatogram showed a spot with the R_F values and specific color of ornithine. The quantity of ornithine was estimated to be 0.01 micromole per gram of leaves. Two-dimensional chromatograms of solutions were prepared from extracts of 10-gram quantities of leaves by preliminary separation on Dowex-50 ion-exchange columns with ammonium hydroxide or hydrochloric acid elution. Subsequent band paper chromatography confirmed the presence of ornithine at this low concentration.

Arginine: One-dimensional chromatograms with and without the addition of authentic arginine and treatment of the paper with the Sakaguchi reagents (1) showed only a single spot. Quantitative analyses of the unpurified wheat extract gave a value of 0.77 micromole per gram of leaves.

The quantities of the more common amino acids compared to those of the ornithine cycle are shown in table II.

WATERMELON SEEDLINGS, *Citrulline:* An extract from 10 grams of seedlings treated on a Dowex-50 column with water and hydrochloric acid elution showed the presence of a compound with the properties of citrulline. Paper chromatography of the water eluates from the column and PDB treatment gave no indication of the presence of allantoin. Two-dimensional chromatograms of untreated extract showed a PDB-positive spot with the migration properties of citrulline. Strong reactions with ninhydrin and PDB indicated there were relatively large quantities of the compound in the seedlings. It had R_F values on paper chromatograms in the solvents BAW, cresol and phenol which were identical with those of citrulline. The compound appeared in the appropriate hydrochloric acid fractions from a Dowex-50 column and gave positive reactions with PDB and ninhydrin. The compound reacted as citrulline in the colorimetric method of Knivett (8). A sample that was free from arginine and ornithine was refluxed for 2 hours with 14% $Ba(OH)_2$. After precipitation of the barium, evaporation and re-dissolving in water, the solution was shown to contain ornithine by the vanillin test and by the Chinard colorimetric test (2), thus verifying the presence of citrulline. An unhydrolysed sample, otherwise treated similarly, did not give a reaction for ornithine.

Quantitative analyses of extract from watermelon seedlings by paper chromatography and optical density measurements of the PDB-colored spots showed a citrulline concentration of 0.7 micromole per gram fresh weight. Other seedling samples gave quantities of citrulline of the same order of magnitude.

Ornithine: One- and two-dimensional chromatograms in BAW, phenol, MTW, and cresol showed the presence in watermelon seedling extract of a compound with the R_F values of ornithine and which gave a positive reaction to ninhydrin and vanillin treatments. The compound was eluted from a Dowex-50 column with hydrochloric acid in the fractions which ornithine normally appeared and in the same fractions as added authentic DL-ornithine-2-C^{14}. The compound gave a positive reaction in the Chinard colorimetric test for ornithine. Estimates of the quantity of ornithine based on the size and color of vanillin spots on chromatograms was 0.2 micromole per gram of seedling.

Arginine: One-dimensional chromatograms in BAW of extract representing 70 mg of seedling showed a single Sakaguchi-positive spot with the R_F value of arginine. The presence of arginine was confirmed by the elution pattern from a Dowex-50 column as compared with authentic unlabeled and C^{14}-labeled arginine. A second Sakaguchi-positive compound was observed in the extracts of large quantities of seedlings after treatment on a Dowex-50 column with acid elution. The compound, whose identity was not determined, was eluted in the 4.0 M HCl fractions before arginine and was present in relatively small quantities.

Quantitative analyses for arginine in extract from watermelon seedlings gave values of the order of magnitude of 1 micromole arginine per gram of seedling. Analyses of roots, stems, and cotyledonary leaves gave values of 0.71, 0.70, and 1.2 micromoles arginine per gram of tissue, respectively.

The more common amino acids were identified by paper chromatography, specific spot color, and published elution curves. Some of the amino acids found, with an estimate of the quantities, are recorded in table II. (It was of interest that there was no evidence on a number of two-dimensional chromatograms for the presence of asparagine.)

DISCUSSION

A study of the interconversion of ornithine cycle amino acids in higher plants would be facilitated if plant material was readily available which contained measurable quantities of these compounds.

The only report in the literature describing the presence of ornithine, citrulline and arginine in a single plant was that of Virtanen in the alder (11). This plant was not available to us for study and therefore a number of plants which were readily available were investigated.

The results of the investigations on wheat roots and barley leaves showed that citrulline was present in quantities less than 0.03 micromole per gram fresh weight. The possibility that allantoin was present in these plant materials, as in wheat leaves, complicated the demonstration of the presence of citrulline by paper chromatographic methods. The use of ion-exchange columns in conjunction with paper chromatography, however, showed that small quantities of citrulline were present in wheat leaves. Although the quantitative results were at best approximations, they showed that citrulline was present in wheat leaves in amounts that were only one-tenth to one one-hundredth the quantities of the more common amino acids. On the other hand, the quantities of citrulline in watermelon seedlings were of the same order of magnitude as the more common amino acids.

Ornithine was found in wheat roots and leaves in quantities similar to citrulline. It was not observed in barley leaves. The concentration of ornithine in watermelon seedlings was one-fifth that of the citrulline but was sufficient so that the amino acid could be readily recognized and isolated.

These results confirm earlier reports of the presence of arginine in wheat leaves (12) and roots (6). It was also found in watermelon seedlings. The quantities of this amino acid were similar to the more common amino acids in the plants investigated.

The relatively small quantities of ornithine and citrulline in the wheat plant explain why they have not been recognized previously. In this, as well as other higher plants, the common amino acids are present in quantities of about 1 or 2 micromoles per gram of fresh tissue. A satisfactory chromatogram may be obtained with about 0.1 micromole of each of the amino acids. Ornithine and citrulline were present in about one one-hundredth of these concentrations, however, and a chromatogram contained only about 0.001

micromole or less of these compounds. Such quantities would normally go undetected. If, on the other hand, the aliquot of solution chromatographed was sufficiently large to allow detection of ornithine or citrulline, either the more abundant amino acids would mask these compounds or the paper chromatograms would be unsatisfactory because of overloading.

The occurrence of ornithine, citrulline, and arginine in wheat and watermelon plants suggests that these compounds may have a more widespread occurrence than is recognized at present. The mere presence of these compounds, of course, does not show their function in plant metabolism or elucidate the pathway of their formation. Although urea is a common constituent of plants, it would appear unlikely that any extensive synthesis of urea is taking place. The role a "urea cycle" would play in the plant's metabolism is not immediately apparent. For this reason it is of interest to learn more of the interconversion of these amino acids in the plant and their relationship to other metabolites. The results of the present investigation indicate the suitability of watermelon seedlings for further studies of the ornithine cycle and metabolic studies (7) will be reported upon in more detail.

Summary

1. The occurrence of ornithine, citrulline, and arginine in young seedlings of wheat, barley, and watermelon is compared with the concentration of other amino acids.

2. Although ornithine and citrulline were both found in wheat and barley tissues, their concentration was only one to two percent that of arginine and other more common amino acids in these tissues.

3. In a typical sample of wheat leaves, ornithine and citrulline were present in concentrations of less than 0.01 micromole per gram of fresh material.

4. In watermelon seedlings, these amino acids were present in concentrations comparable with that of arginine and other amino acids.

5. Although large quantities of asparagine were found in wheat leaves, this amino acid was not detected in watermelon seedlings.

6. The possible relationship of ornithine cycle amino acids to each other and to the metabolism of the plant is discussed.

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A PARTICULATE PYRIDINE NUCLEOTIDE OXIDASE SYSTEM OF THE WHEAT EMBRYO[1,2]

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Although mitochondria appear to be the principal site of terminal oxidation in plant tissues, the occurrence of soluble DPNH and TPNH oxidase systems has been described in a number of well-documented reports (1, 4). These non-cytochromal oxidases have been found to be variably sensitive to inhibition by ascorbate, catalase and hydrogen peroxide; and to stimulation by manganous ion.

The purpose of the present communication is to report on the intracellular distribution of non-cytochromal DPNH and TPNH oxidation in viable wheat embryos. It will be shown that about 80 % of such oxidative capacity resides in particles different from mitochondria. These particles oxidize DPNH and TPNH at approximately equal rates, produce H_2O_2, and have peroxidative activity. Of the commonly listed peroxidase acceptors, *p*-aminobenzoic and anthranilic acids have proved to be the most effective.

METHODS

Homogenates of wheat embryos were prepared and fractionated in 2 M sucrose according to a procedure previously outlined (5). DPNH, TPNH and cytochrome oxidase activities were determined spectrophotometrically. Standard procedures were used for manometric assays. DPNH and TPNH were obtained commercially (Sigma Chemical Corporation). *P*-aminobenzoic and anthranilic acids were recrystallized from aqueous alcohol and showed melting points of 186-7, and 145-6° C, respectively. Crystalline aldolase, phosphoglyceraldehyde dehydrogenase and catalase were purchased from Worthington Biochemicals.

[1] Received June 4, 1957.

[2] Contribution No. 361.

RESULTS

The capacity of different subcellular fractions to oxidize either TPNH or DPNH in the absence of added cytochrome c is shown in table I. The pattern of distribution is the same whether measurements are made in the presence or absence of the stimulants, Mn^{++} and *p*-aminobenzoic acid. Except for less than 10 % of the activity in the soluble fraction, the oxidation is uniformly inhibited by traces of ascorbic acid. From these results we conclude that in homogenates of wheat embryos, the bulk of the capacity to oxidize pyridine nucleotide coenzymes independently of the cytochromes is localized in comparatively heavy particles. The activity of the soluble fraction may be due to a parallel partition of enzymes within the intact cell or may result from an autolysis of structures incident to cell damage, a phenomenon noted in Amoebae by Holter (3). If autolysis does affect distribution, then the findings of Conn et al (1) and of Humphreys (4) that extracts of commer-

TABLE I

SUB-CELLULAR DISTRIBUTION OF PYRIDINE NUCLEOTIDE OXIDASE IN HOMOGENATE OF THE WHEAT EMBRYO

FRACTION	PROTEIN N CONTENT (MG)	SPECIFIC OXIDASE ACTIVITIES			TOTAL ACTIVITIES DPNH	PERCENTAGE OF TOTAL	
		DPNH	TPNH	DPNH + *p*-AMINOBENZOATE		PROTEIN NITROGEN	DPNH OXIDASE
Homogenate	177	0.21	0.23	0.82	37.2	...	...
Nuclear (unpurified)	36	0.22	...	0.73	7.9	21	21
Sediment 2	17	0.83	0.89	3.1	14.1	10	37
Sediment 3	18	0.18	0.23	0.58	3.2	11	9
Floating residues	39	0.18	...	0.70	7.0	23	19
Supernatant fluid	51	0.12	0.13	0.60	6.1	30	16
Sums	161	...	...	...	38.3	95	102

The tissue was homogenized in 2 M sucrose and the nuclear fraction spun down at 12,000 × g for 20 min. The remainder was centrifuged for the same time at 140,000 × g to yield sediment 2. Sediment 3 was obtained by diluting the supernatant with an equal volume of water and centrifuging for 20 min at 140,000 × g; this fraction contained most of the mitochondria. The resultant supernatant was still opalescent but was not fractionated further. The "floating residues" were skimmed off the surface of the 2 M sucrose suspension after centrifuging at 12,000 and 144,000 × g. Activities were measured at 25° C by following the changes in optical density at 340 mμ. The reaction mixture contained the following (in micromoles): Sucrose 1400; $MnCl_2$, 0.13; DPNH or TPNH, 3; phosphate buffer (6.8), 150; *p*-aminobenzoate, if included, 50. Final volume = 2.7 ml. Readings were taken every ½ min for 5 min, but activities were calculated from the last 4 min because of a non-enzymatic oxidation occurring in the soluble portion of the tissue during the 1st min of measurement. Activities are expressed as micromoles substrate oxidized/mg N × 1 min.

TABLE II

COMPARATIVE DISTRIBUTION OF ENZYME ACTIVITIES BETWEEN MITOCHONDRIAL FRACTION AND OXIDASE PARTICLES

	DPN CYTOCHROME C REDUCTASE	CYTOCHROME C OXIDASE	DPNH OXIDASE
Mitochondria	2.1	7.6	0.3
Oxidase particles	0.1	0.3	3.3

Activities are expressed as micromoles of cytochrome c oxidized or reduced and as micromoles DPNH oxidized per ml of suspension × 1 min. Cytochrome oxidase was determined spectrophotometrically as previously described (5); cytochrome c reductase activity was determined in presence of 10^{-4} M cyanide using commercially reduced DPNH as substrate. Mitochondria were isolated from a 1 M sucrose suspension (5).

cial germ contain similar enzymes in soluble form could be reconciled with the results reported here.

The oxidase particles can be physically separated from the cytochrome system because they sediment at centrifugal speeds far lower than those required for mitochondria. Whereas 60 % or more of the oxidase may be spun down in 20 minutes from a 2.0 M sucrose solution at 140,000 × g, mitochondria must be suspended in a 1.3 M sucrose solution before they can be thus sedimented. A comparison of oxidase and mitochondrial fractions in terms of enzyme properties illustrates the degree of separation achieved (table II). The oxidase particles can neither reduce cytochrome c nor oxidize its reduced form. Furthermore, Antimycin "A" which at a level of 0.3 μgm/ml inhibits the DPNH-cytochrome c reductase of the mitochondrial fraction has no effect on the DPNH oxidase of these relatively heavy particles.

The relationship of the oxidase system thus isolated to the in situ structures of the cell is obscure. Viewed under the light microscope, the suspension consists of particles which are heterogeneous in size and shape. Uniformity could not be achieved by repeated centrifugations. Nor could the enzymatic activity be correlated with a narrow range of particle size. Particles with the highest specific activity (0.9 micromoles/mg N × 1 minute) sediment from the 2 M sucrose suspension at centrifugal forces between 15,000 and 57,000 × g but this fraction accounts for only 25 % of the total oxidase capacity and activities taper off gradually on either side of this range. The broad distribution may be explained in one of two ways: either there is a random adsorption of enzymes by unrelated subcellular structures or the structures containing the enzymes are of varied size and density under conditions of fractionation.

Adsorption is unlikely though it cannot be excluded. Repeated washings of the particles with 2 M sucrose increases their specific activity and decreases the concentration of unrelated components (table III). Furthermore, as will be discussed later, the oxidase system appears to involve more than one enzyme, and yet all the properties indicative of this multiple constitution—substrate specificity, oxidase, peroxidase and catalase activity—remain, with one exception, in approximately constant proportion among the different fractions of particles. The exception is the supernatant which contains a heat labile component promoting a greater stimulation of DPNH oxidation by *p*-aminobenzoic acid than that occurring in the other fractions. Even so, the near constancy in proportion of activities would be unlikely in a situation of random adsorptions.

The DNA in the oxidase fraction (table III) probably arises from nuclei broken in the course of homogenization. Its partial removal by washing is of particular interest since it is present in concentrations slightly higher than those for intact nuclei (5), and since DNA can be an effective adsorbent of protein. The possibility that the DNA remaining after washing was functionally associated with the oxidase was eliminated by preincubating a suspension of particles with crystalline deoxyribonuclease. The hydrolysis of the DNA was not accompanied by a diminution in oxidative activity. This result was confirmed by adding sodium choleate to the particles. Under the conditions indicated (table III) almost all of the oxidase went into solution whereas most of the DNA remained attached to sedimentable structures. The small amount of dissolved DNA could be precipitated by protamine without altering the activity of the preparation.

The absence of any relationship between DNA and oxidative activity plus the failure of disintegrated

TABLE III

EFFECTS OF VARIOUS TREATMENTS ON COMPOSITION OF OXIDASE PREPARATIONS

TREATMENT	DPNH OXIDASE		TPNH OXIDASE		ALCOHOL DEHYDROGENASE		MG DNA/MG PROTEIN N	
	BEFORE	AFTER	BEFORE	AFTER	BEFORE	AFTER	BEFORE	AFTER
3 Washings in 2 M sucrose	2.6	4.2	2.7	4.2	0.45	0	1.46	0.96
Solubilization with sodium choleate	3.1	4.7	...	...	...	...	2.5	0.075

Enzymatic activities are expressed as micromoles DPNH oxidized/mg N × 1 min in the presence of *p*-aminobenzoate. Alcohol dehydrogenase was measured spectrophotometrically in the presence of 1×10^{-3} M sodium ascorbate to inhibit DPNH oxidation. Twelve micromoles of acetaldehyde were used as substrate. The activities were checked in the presence of 10^{-4} M *p*-chloromercuribenzoate which inhibited the dehydrogenase without affecting the oxidase. Solubilization was carried out by diluting the sucrose to 0.5 M, adding enough sodium choleate to give a concentration of 1 %, and allowing the suspension to stand for ½ hr at 0° C before centrifuging.

nuclei to yield an active oxidase fraction point to a cytoplasmic origin of the particles.

Their microscopic heterogeneity might be due to their presence as such within the cell, or it might arise during homogenization from a breakdown of larger structures. There is no evidence bearing on this point, but it is worth emphasizing that what are here called particles may as likely as not be disintegrated products of a filamentous or membranous network.

Chemically, the properties of the oxidase particles combine those of the soluble wheat germ oxidases described by Conn et al (1), and by Humphreys (4). A notable difference is that of substrate specificity. The system of Conn et al (1) oxidized DPNH at a quarter the rate of TPNH, that of Humphreys was tested only for TPNH. The particles here isolated oxidized both coenzymes at the same rate under conditions of spectrophotometric assay. Since the equality was found in residues obtained at different centrifugal speeds (table I), we infer this property to be a real characteristic of the system. Commercial wheat germ has probably undergone some autolysis in the course of its preparation (6) and this effect combined with the procedures used by the investigators to extract the enzymes, might well result in a partial solubilization of naturally coherent structures. If such structures contain different enzymes for DPNH and TPNH oxidation then either differential solubility or lability could account for the results obtained with extracts of commercial germ. That the oxidative enzymes can be released from the sedimentable elements by means of 1 % sodium choleate has already been demonstrated. The resulting preparation without any further purification has nine-fold the activity of that purified by Conn et al (1). A preparation of the latter containing 0.5 mg of protein caused an optical density change of 0.103 in 5 minutes while under similar conditions particles with an equal protein content cause a change of about 0.900.

In agreement with the observations of Humphreys (4) the system has a requirement for manganous ions (table IV). Zinc, copper, magnesium, ferrous or ferric ions cannot replace manganese as evidenced by their failure to reverse the inhibitory effect of o-phenanthroline (fig 1). A variety of other metal-complexing agents are inhibitory; of these, sodium versenate (5×10^{-5} M) and cyanide (1×10^{-5} M) appear to be the most effective.

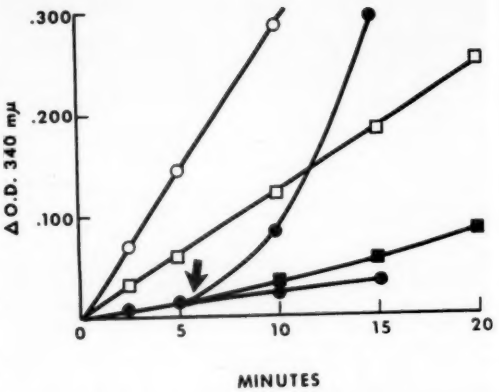

FIG. 1. The catalytic effect of $MnCl_2$ on DPNH oxidation. Measurements were made on particles suspended in the basic medium referred to in table I with indicated modifications. Open circles: complete medium. Solid circles: $MnCl_2$ omitted but *o*-phenanthroline (1.2×10^{-5} M) present. Upper of two curves shows change occurring upon addition of $MnCl_2$ (arrow) to a concentration of 2.4×10^{-5} M. Open squares: no $MnCl_2$ but 1.2×10^{-5} M *o*-phenanthroline plus 5×10^{-5} M ferrous sulphate present before addition of particles. Solid squares: no $MnCl_2$ but 1.2×10^{-5} M *o*-phenanthroline with 5×10^{-5} M ferrous sulphate added at arrow.

TABLE IV

STIMULATION OF DPNH OXIDATION BY $MnCl_2$ AND *p*-AMINOBENZOIC ACID (PABA)

ADDITIONS	ΔO.D.$_{340\ m\mu}$/5 MIN
None	0.140
3×10^{-5} M $MnCl_2$	0.240
1.6×10^{-3} M Paba	0.220
$MnCl_2 + 10^{-3}$ M Paba	0.450
$MnCl_2 + 1.6 \times 10^{-2}$ M Paba	0.900

The conditions of oxidase measurement were the same as those described in table I except for the omission of $MnCl_2$ in the basic medium. The oxidase preparation used was similar to that listed as "Sediment 2."

The sensitivity of the oxidase system to inhibition by reducing agents has also been noted. This is somewhat in line with the observation of Humphreys (4) on an unidentified cofactor which lost its stimulating effect if reduced by ascorbic acid (but not by hydroxylamine). A variety of reducing substances—hydroquinone, glutathione, hydroxylamine, ascorbic acid—inhibit oxidation by the particles, but of these, ascorbic acid is the most effective (5×10^{-7} M) and probably the most revealing. If the curves in fig 2 are followed it can be seen that in the presence of any residual oxidation of DPNH the ascorbic acid is gradually oxidized and its inhibitory effect destroyed. In the presence of relatively high concentrations of ascorbic acid (10^{-4} M), there is no residual oxidation and the inhibition persists unless H_2O_2 or permanganate is added to the medium. At marginal concentrations, the gradual oxidation of ascorbate may be prevented by addition of crystalline catalase to the system (fig 2).

The formation of H_2O_2 in the course of coenzyme oxidation is not only suggested by the effectiveness of catalase in maintaining ascorbate inhibition but can be directly demonstrated by means of a peroxidase acceptor, such as *p*-aminobenzoic acid. The latter compound is unaffected by H_2O_2 alone but turns red in the presence of a peroxidase (7). Since the par-

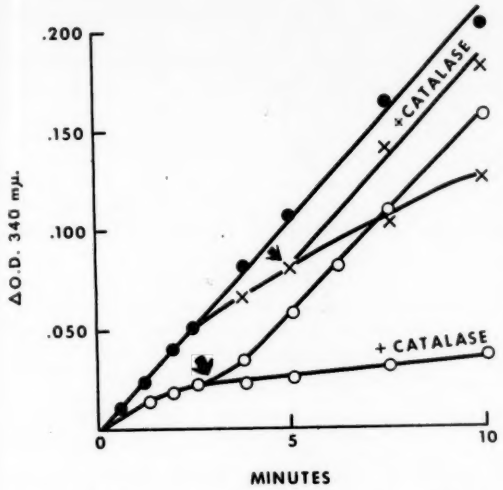

FIG. 2. Effects of ascorbate, catalase and H_2O_2 on DPNH oxidase. All tests were performed in the presence of 5×10^{-5} M $MnCl_2$ but without *p*-aminobenzoic acid. Solid circles: Control assay in the presence or absence of 2 μl commercial crystalline catalase suspension (Worthington, 5000 units/ml). Open circles: 5×10^{-7} M ascorbic acid, plus 2 μl of catalase added at arrow. Crosses: 1×10^{-4} M H_2O_2, + 2 μl of catalase added at arrow.

ticles have peroxidative activity it is only necessary to make available sufficient quantities of reduced DPN to cause detectable coloration. This is achieved under the conditions used for manometric assays (table V). The amount of red *p*-aminobenzoic acid formed, however, is not equivalent to the H_2O_2 presumed to arise from coenzyme oxidation. In the presence of a commercial peroxidase preparation, 0.5 micromoles of H_2O_2 produces an easily measurable concentration of red compound, but 0.5 micromoles of DPNH oxidized by the particles in presence of *p*-aminobenzoic acid produces no detectable color even though its addition quadruples the rate of oxidation (table IV). The low yield of red product may be due to its secondary reduction but whether the observed reaction is an enzymatically governed one has not been determined. Stimulation of pyridine nucleotide oxidation by *p*-aminobenzoic acid may be without a real counterpart in the intact wheat embryo. Most of the listed peroxidase acceptors (9) do not stimulate DPNH oxidation. Mono- and di-amines are inhibitory (hydroxylamine is effective at 1×10^{-5} M); the phenols—*p*-cresol, resorcinol and catechol—cause a rapid oxidation of DPNH in absence of tissue and therefore cannot be tested; 2,6-dichlorophenol-indophenol and hydroquinone are inhibitory; benzoic acid, tyrosine and tryptophane are without effect.

Free H_2O_2 is probably not an intermediate in the reaction although such a mechanism has been suggested for one of the soluble systems (1). Micromolar amounts of H_2O_2 either inhibit oxidation or remain ineffective (fig 2). Only in the presence of an inhibitory reducing substance (e.g., ascorbic acid) has the addition of H_2O_2 been found to stimulate oxidation. Furthermore, catalase of sufficient strength to remove any free H_2O_2 in the system (fig 2) does not depress the rate of oxidation. If, however, the catalase concentration is multiplied by five or more the oxidation is inhibited. The effect is not removed by prolonged dialysis of the catalase and is destroyed by heating. Inhibition of soluble TPNH oxidase by added catalase has been noted by Conn et al (1) and this was regarded as partial evidence for the participation of the H_2O_2 in coenzyme oxidation. The requirement here found for a comparatively high concentration of catalase to effect inhibition suggests otherwise. Some component of the reaction system is susceptible to complexing with added catalase. It may well be that the component is a bound peroxide requiring an endogenous peroxidase or catalase to complete the oxidation sequence.

Manometric determinations of oxidase activity give appreciably lower values than those given by spectrophotometric assays (table V). The reasons for this are undetermined. There is no evidence of a failure in the DPNH generating system as its activity was checked in the presence of the particles by adding ascorbic acid to inhibit the oxidase. Even allowing for catalase activity which would release half the oxygen consumed in peroxide formation, the amount of DPNH oxidation measured spectrophotometrically would require twice the oxygen consumption observed. It is possible that the particles become partly inactivated when incubated for the periods of time (30 minutes) required for manometric assays, but if such inactivation does occur it is not observable as a progressive fall in rate of oxygen consumption. The latter is approximately constant for the first 20 minutes of measurement.

TABLE V

MANOMETRIC MEASUREMENTS OF OXIDASE ACTIVITY

ADDITION	MICROATOMS O_2 CONSUMED/MIN
Anthranilic acid	0.0
Dehydrogenase	0.03
p-Aminobenzoic acid + dehydrogenase	0.11
Anthranilic acid + dehydrogenase	0.23

Determinations were made at 25° C in standard Warburg vessels. The composition of the basic medium was as follows (in micromoles): Sucrose, 600; sodium arsenate buffer (pH 7.0), 100; $MnCl_2$, 0.2; hexose diphosphate, 32; DPN, 0.1, and a non-limiting amount of crystalline aldolase. Anthranilic and *p*-aminobenzoic acid were each supplied in 100 micromolar amounts; the crystalline dehydrogenase added was sufficient to reduce 0.5 micromoles of DPN per min. The amount of particles added to each flask was, according to spectrophotometric assay, capable of oxidizing 0.28 micromole DPNH/min in presence of *p*-aminobenzoic acid. Final volume = 2.5 ml.

Discussion

Under conditions of spectrophotometric assay the capacity of homogenized wheat embryos to oxidize DPNH or TPNH via an ascorbate sensitive pathway in the absence of peroxidase acceptor is 0.22 micromoles/mg N × 1 minute. This value is equivalent to the oxidation of 0.44 micromoles of cytochrome c. It is of interest, therefore, that under similar conditions of assay wheat embryo homogenates oxidize 2.0 micromoles of cytochrome c/mg N × 1 minute (5). The capacity of the oxidase is thus 20 % that of the cytochrome system. Allowing for the hazards of interpreting comparative in vitro activities, the capacity of this non-cytochromal oxidase system cannot be regarded as negligible. The fact that cytochrome c is present in limiting amounts in the ungerminated embryo (2) may add to the importance of this alternative channel of coenzyme oxidation during the early stages of embryonic growth.

The morphology of the structures which incorporate this oxidase system remains unrevealed. It is reasonably certain that the structures are not mitochondria. They may be discrete particles but there is no evidence compelling such a conclusion; it is equally possible that they are products of a mechanical breakdown of larger cytoplasmic structures. It would appear that the association between the oxidative moiety and the physical skeleton of the system is of a much looser kind than that existing between cytochrome oxidase and the mitochondrial framework. Sodium choleate solubilizes the pyridine nucleotide oxidases leaving the sedimentable components without oxidative activity, whereas under similar treatment cytochrome oxidase remains attached to the mitochondrial membrane (8).

Summary

In homogenates of viable wheat embryos 80 % of the capacity to oxidize DPNH and TPNH independently of the cytochrome system is localized in particulate structures. These particles are readily separated from the mitochondria by differential centrifugation.

The oxidase system has properties in common with those previously described for soluble oxidase preparations. It has a specific requirement for Mn^{++}, and is inhibited by catalase and a variety of reducing agents, most effectively by ascorbic acid. H_2O_2 is a reaction product, which in the presence of an appropriate acceptor (*p*-aminobenzoic and anthranilic acids) is reduced by the peroxidase component of the system.

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NICOTINE PRODUCTION AND GROWTH OF EXCISED TOBACCO ROOT CULTURES[1,2]

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Experiments with interspecific grafts have shown that, despite its lesser mass, the tobacco root produces much greater quantities of nicotine than does the aerial shoot (cf 4, 7, and 9 for review). Possible explanations for this inequality include (a) a gross difference in the intrinsic rates of nicotine synthesis of root and shoot, and (b) an unequal distribution between the major plant organs of the total mass of tissue responsible for synthesis. If, as has been suggested, the growing tips of root and stem were major loci of nicotine formation much of the difference could be explained by parallel differences in the numbers of such tips, the advantage obviously resting with the root system. Available evidence is insufficient to support either hypothesis.

The idea of concentration of alkaloid synthetic

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activity in the growing root and shoot tips is not new. Early experiments of Schmidt and Serrano (11) showed a direct relationship between the total nicotine content of the plant and the protein content of the root. Laschuk (8) observed a close parallelism between alkaloid production and the development of roots on intact tobacco plants. He concluded that the production of nicotine is dependent upon continued growth and development of the root tip and not upon the general metabolic activities of root tissues as such. Histochemical evidence demonstrates that nicotine appears first in the shoot tip as well as the root tip of germinating tobacco seeds which are initially alkaloid free (2, 6, 10). However, histochemical evidence is incapable of providing a distinction between loci of synthesis and loci of accumulation from a translocation stream.

A more detailed examination of nicotine production and growth was undertaken in the hope of learning whether or not there is a close correlation between the two. For study of the root, suitable experimental material was found in the excised root culture of *Nicotiana Tabacum* var. Turkish. This material possesses certain important characteristics: 1) There is no discernible secondary growth in root diameter (see below). Hence, it was possible to compare relative capacities for alkaloid synthesis of growing root tips and of matured regions of the primary root uncomplicated by the possibility that appreciable rates of such synthesis might occur in tissues of secondary origin (e.g., an active cambium). 2) The roots can be subcultured through an indefinite number of short-term passages. The clones thus established possess relative genetic stability. 3) The physical and chemical environment of the cultures can be controlled easily inasmuch as growth occurs in the dark and in a medium which is entirely synthetic except for a small amount of yeast extract. Physiological stability is thus also a possibility. 4) Nicotine is the only alkaloid that we have detected in properly prepared extracts of these roots.

As a result of this combination of circumstances, it has been possible to study the interrelations of growth[3] and nicotine production under essentially steady state conditions with respect to the individual root branch.

Materials and Methods

Root tips were isolated aseptically from two seedlings of *Nicotiana Tabacum* L. var. Turkish and cultured as separate clones in a medium modified from that described by White (13) by the addition of 0.01 ppm copper and 0.003 ppm molybdenum. In earlier experiments the pH was adjusted to 5.6 but this was found later to be unnecessary. The cultures were grown in 125-ml Erlenmeyer flasks containing 20 to 40 ml of autoclaved medium depending on the duration of the experiment. Except where otherwise stated each flask was inoculated with a single root tip approximately 10 mm long. Care was taken to use only secondary tips from rapidly growing cultures of uniform size. All cultures were grown in the dark at a temperature of $29 \pm 0.5°$ C.

Nicotine was assayed in steam distillates by techniques (5) modified for increased sensitivity from those of Willits et al (14). Recovery of nicotine from twenty aliquots of a stock solution of the pure alkaloid by these methods averaged 99.4 ± 1.4 %. Since the root in culture has no aerial shoot to which the nicotine may be transported, the latter is excreted presumably through the cut end into the surrounding medium. This necessitates separate analyses of both root and medium. At harvest the root tissue was dried at 70° C and the pooled medium was concentrated in vacuo. Portions of each were separately steam-distilled for nicotine assay.

Results

Data on growth measurements and nicotine accumulation under standard conditions are found in table I. Cultures originally containing 30 ml of medium and 1 root tip were harvested at intervals to the 20th day. At the end of 20 days the surface of the medium was completely covered with a dense mat of intertwined branches which caused extreme difficulty in separation for purposes of length measurements. Consequently most experiments were terminated at this time.

Excised root cultures accumulate nicotine as they grow. When measurements are confined to the exponential phase of growth, plots of length, dry weight and nicotine against elapsed time show similar slopes.

Table I

Growth and Nicotine Production by a Clone of Excised Roots of Nicotiana Tabacum var. Turkish

Expt	Age of culture, days	No. of cultures	Mean dry wt mg	Mean length, mm	Mean no. of branches*	Mean nicotine yield, μgm
A	7	30	0.34	56	1.6	11
	11	27	0.67	99	4.6	23
	14	20	1.46	231	13.3	50
	17	10	2.86	488	29.7	91
	20	10	4.77	744	47.2	139
B	7	28	0.32	57	3.4	9
	11	22	0.89	163	7.5	26
	14	23	1.71	284	11.8	55
	17	12	3.27	564	33.2	110
	20	7	6.34	1098	73.5	182
C	7	18	0.36	...	...	10
	7	18	0.43	...	...	12
	14	14	2.12	...	...	70
	14	14	2.33	...	...	79
	20	15	9.38	...	...	294
	20	13	6.83	...	...	208

* Does not include main branch.

[3] Growth is defined as increase in root volume and is estimated in terms of one or more functions of volume, viz length, dry weight, etc. The relationship between length and dry weight appears in the discussion.

TABLE II

NICOTINE PRODUCTION, GROWTH AND RESPIRATORY ACTIVITY OF EXCISED ROOT SEGMENTS OF NICOTIANA TABACUM VAR. TURKISH DURING CULTURE FOR THE PERIODS INDICATED

SEGMENT TYPE AND TREATMENT	INITIAL			1 WEEK			2 WEEKS	
	NICOTINE	DRY WT	QO_2*	NICOTINE	DRY WT	QO_2*	NICOTINE	DRY WT
	μgm	*mg*		*μgm*	*mg*		*μgm*	*mg*
Mature segments	286	18.8	4.7	284	17.8	1.3	....	....
Tip segments	65	3.0	12.4	773	30.0	..	4437	130.0
Tip segments with 10^{-6} M IAA	...	...	...	134	15.4	5.9	147	29.0

* Microliter oxygen absorbed per mg dry tissue per hr.

This also means that a straight-line relationship exists between any pair of these dependent variables. Details of the statistical analysis of the data are presented in the discussion. Due to the more rapid growth obtained in experiment (b) the data from the 20-day harvest were eliminated from the statistical analysis. Under our conditions accurate measurement becomes almost impossible when total root length exceeds 800 mm. In addition, it appears from the few such cases so far analyzed that the effects of progressive diminution in nutrient supply make themselves felt at about this stage of culture development.

Differences in nicotine synthetic capacity between mature portions of the root and the root tips were demonstrated as follows. Two-hundred segments (5 per flask and 5 mm long of clone 1) of each were cultured separately in the usual medium. The mature sections were taken from the oldest part of the culture in order to eliminate the possibility of formation of new root branches. The averaged results of eight replications taken initially and after one week of culture are shown in table II. After one week the root tips had increased their alkaloid content as well as their dry weight approximately ten-fold. This increase could not be attributed to root branches since they did not appear until after the first week. On the other hand the older sections gained neither in dry weight nor in alkaloid content. Although growth did not occur in the segments there was still evidence of metabolic activity as shown by the respiratory gas exchange data contained in table II.

The observed connection between nicotine production and growth was examined under a different set of conditions. A series of concentrations of 3-indolylacetic acid (IAA) was provided in the standard culture medium. It was found that, at 10^{-6} M, linear growth was almost completely inhibited with the preservation of otherwise normal root appearance (i.e., absence of hypertrophy, etc.),

Four separate experiments were performed in which five root tips were placed in each of 50 culture flasks containing 20 ml complete nutrient solution 10^{-6} M with respect to IAA. Means for dry weight and nicotine production are contained in table II. During the first week, increase in dry weight was reduced 50 % by IAA although growth in length appeared to have been virtually halted by this compound. The corresponding yield of nicotine was reduced to 16 % of the control cultures. Changes during the second week were associated with the development of many branch roots in the control cultures. Dry weight of the latter increased 4.3-fold while the corresponding change in the treated cultures was 1.9-fold. IAA therefore decreased dry weight to 14 % of the control yields and nicotine production to 0.36 %. The respiratory gas exchange data are presented to give an index of metabolic activity in the treated roots.

It appears from these results that there is no simple relationship between dry weight and nicotine production when IAA is present. However this conclusion is complicated by the microscopic observation that large quantities of starch were stored in the treated

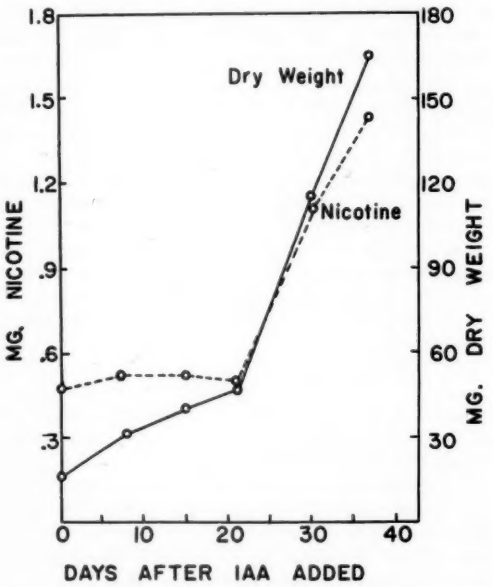

FIG. 1. Relationship between nicotine production and dry wt accumulation at various intervals after addition of IAA to sterile cultures of excised roots of *Nicotiana Tabacum* var. Turkish. Initial concentration of IAA in each case was 10^{-6} M.

roots. None was observed in the control roots. Further data were provided by the following experiment.

Six groups of 25 cultures, each flask containing 5 root tips and 20 ml of medium, were allowed to grow for 1 week before IAA was added to make a final concentration of 10^{-6} M. The groups were harvested consecutively. The results are given in figure 1. For the first three weeks dry weight increased at a slow rate while nicotine synthesis and elongation had apparently halted. This is in agreement with results in table II. Between the third and fourth weeks, these roots, which had shown little, if any, elongation up to that time, began to form many branch roots which elongated normally. Simultaneously, rapid increases in nicotine production and dry weight were observed. The cause of the suddenly renewed elongation was not completely investigated. There are two possibilities. First, there was some evidence that the roots might have become adapted to IAA, since on transferrence to medium without IAA they grew slowly. Second, the IAA may have been oxidized or destroyed by some other means. Whatever the cause, in the absence of elongation nicotine synthesis did not occur, while on resumption of elongation nicotine synthesis continued at a normal rate.

Discussion

A clone of excised tobacco roots can be subcultured through an indefinite number of passages provided secondary tips are used to inoculate the new cultures. These roots produce nicotine as they grow (3). When the duration of each passage is relatively short (less than 21 days), and when tips 10 mm in length taken from actively growing cultures are used for establishing each new passage, the following statements may be made which characterize the growth of the Turkish strain of *Nicotiana Tabacum* employed in our experiments:

(a) There was no discernible secondary increase in root diameter; hence, growth may be considered as essentially a matter of linear root extension. The line of least squares relating root length to dry weight during 20 days of growth possessed a slope of 5.95 μgm per mm with a standard error of estimate ($t = 0.05$) equal to $\pm$ 0.29 μgm per mm (table I). Assuming that appreciable secondary increase in diameter of older roots would have been associated with an axially asymmetric distribution of dry weight, it is clear that little or no secondary growth in diameter did occur.

(b) As the roots grew they also branched. Hence, the number of growing tips increased with age of culture. If the total root lengths of cultures harvested at different ages (table I) are related to numbers of root branches, there is obtained a second linear regression with slope 16.4 mm per branch (or per tip) with a standard error of estimate $\pm$ 1.12 ($t = 0.05$). There is thus no indication of gross inconstancy in developmental pattern during the growth periods employed.

(c) The total linear growth of the entire root with all its branches followed first order kinetics with respect to time, the rate constant being 0.2107 mm per day per mm. Although actually a discontinuous phenomenon, branch production also follows a first order trend of 0.227 branch per day per branch. Virtual equality of rate constants for branch production and for total linear extent implies constancy of growth rate of the individual branch. This justifies the claim for steady state conditions (see above).

The relationships of nicotine production in the same 20-day period to both length and dry weight can be expressed by simple proportionalities. The roots produced nicotine at a rate of 0.191 μgm per mm with a standard error of estimate of $\pm$ 0.010 ($t = 0.05$). Under the standardized conditions of our experiments nicotine production was also related proportionally to dry weight. The rate was 29.05 μgm per mg with a standard error of estimate of $\pm$ 2.14. Limited dry weight increase is possible without accompanying alkaloid formation (fig 1) providing the standard conditions of growth are varied. Hence, the above mentioned rates were dependent upon careful elimination of variations in conditions of culture. Some of these variations are the purity of distilled water, deterioration of the thiamine-glycine stock solution of White (13) during storage, and the presence of toxic contaminants in the salts and sucrose used in preparing the medium. The last-mentioned item is particularly troublesome and has been eliminated only by systematic pretesting of newly acquired stocks against acquisitions of known efficacy.

The close connection between growth in terms of increase in length (or dry weight) and nicotine yield suggests a relationship between the process of alkaloid production and one or more activties of the growing root tip. Dissection of the root into tip and mature region confirms that the alkaloid is synthesized only when tips are present. Therefore, it seems probable that nicotine production depends upon some activity in the root tip rather than upon the general metabolic activities of the entire excised root.

The use of inhibiting concentrations of IAA further shows that the root tip will not synthesize nicotine when it is not elongating. The results obtained from this treatment raise an interesting question. Burström (1) reports that "Supraoptimal concentrations of indoleacetic acid (IAA) inhibit root growth by reducing cell elongation with only small changes in cell multiplication." This has been confirmed by Street et al (12) for excised tomato roots. Microscopic observations of the treated roots in our experiments revealed an accumulation at the tips of many small cells. A comparative histological investigation is needed to ascertain quantitatively the effects of IAA upon rates of cell division and exact limits to the increase in cell volume. The question is whether there were enough cell divisions in the presence of IAA to permit analytical detection of the nicotine formed if division and synthesis are closely related. If there were, this approach can be used to decide which

principal cellular activity of the root tip, division or normal elongation and differentiation, is correlated more closely with nicotine production.

Summary

Excised roots of Turkish tobacco were grown in sterile culture, and a detailed study was made of the interrelations of their growth and nicotine production. During the exponential phase of growth the roots produced nicotine at a constant rate of 0.191 μgm per mm length or 29.05 μgm per mg dry weight. When the growing tips and mature regions of the root were cultured separately, nicotine increases resulted only in the case of the tips. By the use of indoleacetic acid in proper concentration, nicotine synthesis was observed only in those roots where elongation occurred.

Grateful appreciation is expressed for the counsel of Professor R. F. Dawson of Columbia University.

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NICOTINE PRODUCTION AND GROWTH OF TOBACCO SCIONS ON TOMATO ROOTSTOCKS[1,2]

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Although the primary role of the tobacco root in nicotine biosynthesis was established more than a decade ago there has been little investigation of the limited alkaloid productivity of the shoot (cf Mothes (8) for review).

Indications that the tobacco shoot may produce some steam-volatile alkaloid are evident in the earlier data of Dawson (2) where increases in this fraction took place slowly during growth of Turkish tobacco scions on tomato rootstocks. The magnitude of the change was 7.3 mg (calculated as nicotine) per scion in 84 days of growth or 0.017 mg/gm increase in fresh weight. However, the method of assay then employed was neither sensitive nor specific for pyridine alkaloids, and attempts to isolate the alkaloid were unsuccessful.

Again, working at or near the limits of sensitivity of available methods for nicotine assay, Mothes (7) reported that growing leaves, when excised and provided with a good nitrogen supply, accumulated small amounts of alkaloid. Later, Mothes and Romeike (9, 10) found traces of alkaloid in *Nicotiana rustica* scions grafted on tomato.

Similarly, Mashkovtsev and Sirotenko (6) grafted tobacco embryos almost completely devoid of nicotine on tomato rootstocks. By direct isolation as the crystalline dipicrate, a 100,000-fold increase in nicotine content was demonstrated during the growth and development of a single scion. From these and related data they concluded that the characteristic of nicotine synthesis is possessed by any cell of the above-ground part of *Nicotiana Tabacum*.

In this paper we wish to present further data in support of the hypothesis that limited alkaloid production occurs in the tobacco shoot and to describe the patterns of such alkaloid accumulation in relation to growth of the plant.

Materials and Methods

Grafts were made using three different tobaccos: *Nicotiana rustica*, var. *brasilia*, *N. Tabacum* var. Turkish, and *N. Tabacum* var. Connecticut 49. These scions were cleft-grafted with stocks of tomato, *Lycopersicum esculentum* var. Marglobe, and grown

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[2] Submitted in partial fulfillment of the requirements of the degree of Doctor of Philosophy, in the Faculty of Pure Science, Columbia University. Part of this paper is based on work performed under Atomic Energy Contract No. AT(30-1)-1778.

in the greenhouse. Since the first shoots to develop from such grafts would be expected to contain much of the nicotine originally transferred with the scion (2) these were cut back and one axillary bud allowed to develop. Plants of each kind were harvested at four different stages of development. Each lot contained 4 plants which were chosen for similarities in height, and number and size of leaves. The tissues were dried in a forced-draft, hot-air oven at about 70° C.

Cleft-grafts were also prepared between the following varieties of *N. Tabacum* as scions and various Solanaceae as rootstocks: Burley and tomato, Turkish and both tomato and Jimsonweed (*Datura Stramonium*), and Pennsylvania Seedleaf and both tomato and eggplant (*Solanum Melongena*).

Stem callus cultures of *N. Tabacum* var. Turkish were started from the internodes of the upper portions (about 2 to 4 inches from the tip of the stem) of grafted scions after the manner of Skoog and Tsui (12). Stem quadrants 1 cm long were placed on the surface of agar slants prepared from White's medium (14) modified to contain 0.01 ppm of copper, 0.003 ppm molybdenum, 0.5 ppm naphthaleneacetic acid and 0.6 % washed agar. The pH was adjusted to 5.2 before autoclaving for 10 minutes at 15 lbs pressure. The cultures were incubated at room temperature under daylight fluorescent lamps.

Total pyridine alkaloid was assayed directly in the steam distillates of dried plant material weighing up to 3 gm by methods of Wilits et al (15) as modified in this laboratory for increased sensitivity (4). Larger samples weighing up to 10 gm were first thoroughly mixed with 3 gm of $Ca(OH)_2$ and 10 ml of 1.25 *N* NaOH and then extracted for 16 hours with ethyl ether in a continuous Soxhlet extractor. Subsequently, ether was removed by distillation over a small volume of *N* HCl and the aqueous solution remaining was neutralized with NaOH and steam distilled. The alkaloid was calculated as nicotine. Nicotine was also isolated as the dipicrate and identified by melting point, by paper chromatography (11), and by ultraviolet spectrophotometry.

Results

Evidence for Nicotine Production in Scions: Analyses of scion shoots of three varieties of *Nicotiana Tabacum* grafted on various Solanaceae as root-

Table I

Nicotine Concentration (as % of dry wt) of Tobacco Scions Grown upon Solanaceous Rootstocks. (Concentration in the Ungrafted Stock Species is Also Given)

Scion	% Nicotine	Stock species	% Nicotine
Nicotiana Tabacum:		*Solanum Melongena* (Eggplant)	0.0011 **
1. Pennsylvania Seedleaf	0.048		
2. Pennsylvania Seedleaf	0.033	*Solanum Melongena* (Eggplant)	0.0011 **
3. Pennsylvania Seedleaf	0.038 †	*Lycopersicum esculentum* (Tomato)	0.0015
4. Turkish (Samsun type)	0.040	*Datura Stramonium* (Jimsonweed)	0.0005 *
5. Turkish (Samsun type)	0.025 †	*Lycopersicum esculentum* (Tomato)	0.0015
6. Turkish (Samsun type)	0.014	*Lycopersicum esculentum* (Tomato)	0.0015
7. Burley (Low Nicotine)	0.007	*Lycopersicum esculentum* (Tomato)	0.0015

* Data of Wahl (13).

** Total steam-volatile pyridine alkaloid calculated as nicotine.

† Isolated as the crystalline dipicrate and identified as nicotine by melting point.

Table II

Total Nicotine and Dry Weight in the Leaves, Stems and Inflorescences of Three Tobaccos Grown as Scions on Tomato Rootstocks. (Plants Were Harvested at Four Different Stages of Growth.)

Scion	Age	Leaf		Stem		Inflorescence		Total		% Nicotine of dry wt
		Dry wt	Nicotine	Dry wt	Nicotine	Dry wt	Nicotine	Dry wt	Nicotine	
	days	*gm*	*μgm*	*gm*	*μgm*	*gm*	*μgm*	*gm*	*μgm*	
N. Tabacum var. Turkish	21	3.22	1075	0.91	730	0	0	4.13	1805	0.043
	34	18.36	2183	....	....	0	0	....	.....	....
	45	20.45	3025	13.96	5633	1.87	232	36.28	8890	0.024
	62	29.19	3768	30.75	8200	23.71	1202	83.65	13170	0.016
N. Tabacum var. Conn. 49	24	4.96	324	1.44	295	0	0	5.40	619	0.011
	32	10.16	722	3.93	643	0	0	14.09	1370	0.010
	52	30.80	687	15.66	2387	0	0	46.46	3074	0.006
	70	44.46	1506	46.36	3079	4.78	0	95.60	4585	0.005
N. rustica var. *brasilia*	24	4.73	170	1.62	217	0	0	6.35	387	0.006
	42	17.11	364	19.18	504	0	0	36.29	868	0.002
	52	27.95	697	19.25	462	0.76	38	47.20	1159	0.002
	72	38.15	Trace	39.17	0	33.75	0	111.97	Trace	0.000

stocks showed a range of nicotine content from 0.007 % of dry weight in a low-nicotine Burley to 0.048 % in Pennsylvania Seedleaf (table I). From two of these samples nicotine was obtained as the dipicrate with a correct melting point. Plants were included from both greenhouse and field cultures. The concentration of nicotine in tobacco scions grown on tomato (Marglobe) rootstocks and harvested at various stages of scion development varied with the age of the plant (table II). For example, the concentration of alkaloid in a young scion was 2 to 3 times greater than in a mature scion with seedpods. Therefore, although the total nicotine content continued to increase throughout its growth, total dry weight of the scion increased at a faster rate.

The meaning of such data was questioned in 1953 when Wahl (13) reported the isolation of nicotine in very small amounts (1 to 2 mg per 100 gm dry weight) from intact tomato plants. This fact raised the possibility that the nicotine content of the grafted scion was a result of translocation from the tomato rootstock. Examination of the Marglobe variety of tomato in this laboratory revealed a pyridine alkaloid content of 0.0015 % of dry weight (as nicotine) for young plants about 16 inches tall and 0.0014 % for older plants with green fruits. Upon paper chromatography of alkaloid extracts, spots possessing R_f values similar to that of nicotine were obtained. The leaves of eggplant were found to contain a concentration of 0.0011 % alkaloid calculated as nicotine.

A comparison between the concentrations of alkaloid in the tobacco scions and the shoots of intact Solanaceae used as stocks is given in table I. The value, 0.0015 %, for tomato may also be contrasted with the concentrations in tobacco scions given in the last column of table II. With the exception of *N. rustica*, the alkaloid concentration in the tobacco scion is 3 to 40 times greater than that of the stock species despite the greater mass and area of leaf and stem organs of the tobaccos.

The question may be asked whether the intrinsic alkaloid-producing capacity of the tomato rootstock could be substantially greater than indicated by the concentrations found in tomato shoots owing to the occurrence of a relatively high rate of destruction of the alkaloid in shoot tissues. Excised shoots of both tomato and tobacco scions, when cultured with their

TABLE III

NICOTINE CONTENT AND DRY WEIGHT PER SEGMENT OF GRAFTED N. TABACUM VAR. TURKISH STEM PIECES CULTURED ON WHITE'S MEDIUM

WEEKS	NICOTINE μGM	DRY WT, MG
0	3	13
5	110	63
10	158	143
28 *	0	260

* This assay represents only new growth without any of the original stem segment.

TABLE IV

NICOTINE, DRY WEIGHT, AND LENGTH/LEAF OF LEAVES TAKEN FROM SCIONS OF NICOTIANA TABACUM VAR. TURKISH GROWN ON TOMATO ROOTSTOCKS

LEAF NO. FROM BASE OF SCION	NICOTINE, μGM				DRY WT, GM				LENGTH, MM			
	21 DAYS	34	45	62	21	34	45	62	21	34	45	62
1	117	61	72	244	0.34	0.35	0.71	0.58	178	179	240	233
2	137	113	133	236	0.45	0.51	0.64	0.73	202	196	241	265
3	134	99	74	268	0.43	0.70	1.00	0.80	217	249	271	259
4	158	117	108	196	0.48	0.77	0.99	0.97	212	250	278	273
5	133	185	252	277	0.45	1.09	1.05	1.12	202	284	286	287
6	148	180	105	197	0.36	0.97	1.06	1.20	183	279	280	293
7	73	208	188	165	0.26	1.20	0.92	1.14	157	300	266	311
8	71	248	205	190	0.18	1.20	1.15	1.09	135	296	297	306
9	50	120	91	130	0.14	0.97	0.98	1.47	114	292	287	348
10	24	74	301	196	0.07	1.10	1.04	1.31	89	324	294	338
11	21	125	197	145	0.04	0.99	1.19	1.45	70	290	308	346
12	9	119	161	101	0.02	1.01	1.08	1.68	49	310	296	371
13	...	82	157	142	...	1.09	1.08	1.65	...	305	302	362
14	...	75	132	96	...	0.88	1.23	1.58	...	285	311	363
15	...	69	177	184	...	0.97	1.03	1.71	...	291	294	362
16	...	53	165	185	...	0.82	1.14	1.81	...	271	303	360
17	...	52	82	107	...	0.83	0.98	1.84	...	283	284	364
18	...	60	216	228	...	0.76	1.08	1.62	...	272	279	344
19	...	37	54	160	...	0.63	0.80	1.75	...	253	258	346
20	...	34	58	159	...	0.58	0.56	1.38	...	239	225	324
21	...	38	92	101	...	0.39	0.48	1.12	...	195	214	280
22	...	15	0	51	...	0.28	0.23	0.87	...	172	159	252
23	...	14	0	9	...	0.18	0.08	0.26	...	140	106	158
24	...	5	...	0	...	0.09	...	0.06	...	103	...	93

Plants were harvested at 4 stages of growth and each figure represents an average of 4 leaves.

TABLE V

NICOTINE, DRY WEIGHT, AND LENGTH/LEAF OF LEAVES TAKEN FROM SCIONS OF NICOTIANA TABACUM VAR. CONNECTICUT 49 GROWN ON TOMATO ROOTSTOCKS

LEAF NO. FROM BASE OF SCION	NICOTINE, μGM				DRY WT, GM				LENGTH, MM			
	24 DAYS	32	52	70	24	32	52	70	24	32	52	70
1	82	95	41	37	0.48	0.80	0.29	0.84	279	338	205	275
2	39	94	32	33	0.84	0.83	0.52	1.12	367	328	275	330
3	50	135	33	33	0.68	1.16	0.78	1.26	344	383	328	340
4	48	66	29	51	0.68	0.91	1.07	1.40	331	357	375	337
5	24	56	42	33	0.50	1.02	1.08	1.36	280	360	375	332
6	45	80	45	100	0.36	1.01	1.26	1.65	236	361	380	364
7	18	42	29	78	0.23	0.92	1.27	1.91	190	342	377	382
8	10	40	46	131	0.10	0.98	1.41	2.10	123	335	367	394
9	5	42	32	85	0.06	0.82	1.66	2.04	93	308	405	378
10	3	21	41	82	0.03	0.61	1.55	2.13	59	262	394	382
11	..	25	41	95	...	0.48	1.68	2.27	...	237	408	390
12	..	13	32	61	...	0.30	1.97	2.36	...	190	425	396
13	..	7	45	95	...	0.18	1.85	2.20	...	146	408	388
14	..	6	34	72	...	0.10	2.02	2.31	...	110	435	387
15	..	tr	40	40	...	0.04	2.11	2.10	...	73	434	366
16	..	..	33	90	...	...	1.98	2.17	...	...	406	362
17	..	..	28	49	...	...	1.99	2.08	...	...	406	348
18	..	..	30	61	...	...	1.76	1.75	...	...	360	332
19	..	..	14	103	...	...	1.55	1.71	...	...	338	321
20	..	..	11	13	...	...	1.10	1.56	...	...	299	305
21	..	..	9	51	...	...	0.80	1.51	...	...	257	396
22	..	..	tr	23	...	...	0.51	1.35	...	...	211	283
23	..	..	tr	27	...	...	0.31	1.32	...	...	172	280
24	..	..	tr	22	...	...	0.17	1.12	...	...	130	265
25	..	..	0	10	...	...	0.11	1.01	...	...	97	252
26	..	..	..	22	...	...	...	0.88	...	...	...	242
27	..	..	..	9	...	...	...	0.57	...	...	...	205
28	..	..	..	0	...	...	...	0.38	...	...	...	174

Plants were harvested at 4 stages of growth and each figure represents an average of 4 leaves.

cut ends in solutions (1) containing 1.5 mg of nicotine, took up all the alkaloid and, in both cases, converted about 20 % of it to non-steam volatile substances of unknown identity (cf Dawson (3) for significance of these figures). There do not, therefore, appear to exist differential rates of destruction of the translocated alkaloid in tomato and in tobacco.

Nicotinic acid has recently been demonstrated to be one of the immediate precursors of nicotine biosynthesis (5). By utilizing this precursor, further evidence for the production of nicotine in shoots was obtained. Three young Turkish scions from tomato rootstocks were supplied through their cut stems (1) with an ice-cold solution containing 5 mg recoil tritium-labeled nicotinic acid over a period of 8 days. Nicotine was isolated by the sequence: steam distillation into aqueous picric acid containing 8.6 mg inactive nicotine dipicrate, continuous ether extraction from alkaline solution after removal of picric acid, precipitation as oxalate, solution in water, and reprecipitation as the crystalline dipicrate. The yield was 14.5 mg and the specific activity uncorrected for dilution with carrier was 1,056 dpm/mg picrate. The specific activity of the nicotinic acid was 265,000 dpm /mg. Without correction for incomplete uptake of nicotinic acid, for small losses generally incurred during continuous ether extraction, for dilution with carrier, and for amount of preformed nicotine in the shoots, these figures represent a radio-chemical yield of 1.2 %. It is thus evident that the shoot is capable of making nicotine from its precursors. Analytical evidence (3) shows that shoots of this variety in cut stem culture generally lose small amounts of alkaloid. This experiment indicates that such losses may be somewhat greater than realized owing to the occurrence of small amounts of synthetic activity.

Additional evidence for the production of nicotine by aerial organs was obtained from the sterile culture of excised stem segments of *N. Tabacum* var. Turkish. Stem quadrants of grafted Turkish scions were allowed to proliferate callus in sterile culture. Twenty cultures were harvested at 5 and 10 weeks (table III). Both nicotine and dry weight increased but the former did so at a much faster rate. Growth after 10 weeks was poor and the cultures started to turn brown. New growth was then separated from the original stem segment. Assays made at 28 weeks, after several subcultures of the new growth, revealed no nicotine.

The nicotine synthesizing capacities of a vigorously growing callus from *N. glutinosa* were also examined. Again a relatively high nicotine content attained within the first passage was followed by declining amounts in the second and third passages. After the fourth passage, although the cultures were routinely

subcultured for over two years, there was no further indication of alkaloid production even when large quantities of dry tissue (10 gm) were extracted and the extracts concentrated and chromatographed on paper sheets.

Patterns of Nicotine Accumulation in Grafted Scions: The nicotine accumulation pattern in shoots of three species of tobacco grown as scions on tomato rootstocks was investigated by harvesting one lot of four plants of equal size and maturity at four different stages of growth. The material so collected represented the second regeneration from axillary buds on the original grafted scion piece. The pooled leaves of the four plants were analyzed according to their stalk position, with number 1 designated as the first above the graft union. Stems were assayed in their entirety or divided into segments 162 mm long. The inflorescence was also analyzed.

Total nicotine in both varieties of *N. Tabacum* increased rapidly at first and then progressively more slowly as the inflorescences developed (table II). In the initial harvests more than half of the nicotine was contained in the leaves. After all the leaves of the plant were formed, alkaloid accumulated in greatest amounts in the stem so that by the time fruits appeared the stems contained twice as much as the leaves. The inflorescence of Connecticut 49 contained no nicotine, while in Turkish nicotine was found in the inflorescence leaves, stems and even a small amount in the green seed pods.

N. rustica deviated from the above pattern in two ways. First, the nicotine increases were extremely small even though the dry weight increases resembled those of Connecticut 49 in degree. Second, practically all traces of steam volatile alkaloid disappeared from the plants at the fruiting stage.

An examination of the leaves from each stalk position indicated that in spite of fluctuations there was a general trend toward accumulation of nicotine during the growth of the leaf (tables IV, V, and VI). The variation was most evident in the lower leaves which had reached their mature size early in the experiment. Dry weight and length data are presented to give an index of growth.

In Turkish leaves it can be observed that initially the highest amounts of nicotine were found in the basal leaves which also showed the greatest dry weight and length. In the second collection, the nicotine peak had shifted to leaf number 8. In the third, there was no pronounced peak but rather a plateau extending from the 5th to the 18th leaf. There was little change in the final crop except for an increase in the basal leaves, probably the result of translocation from the basal part of the stem (see below). Comparing nicotine content with attained size indicates that there was no relation to either dry weight or length except in the cases of young leaves (first harvest and top leaves of second harvest). In general, the larger leaves did contain greater amounts but there was a wide variation among leaves of the same size.

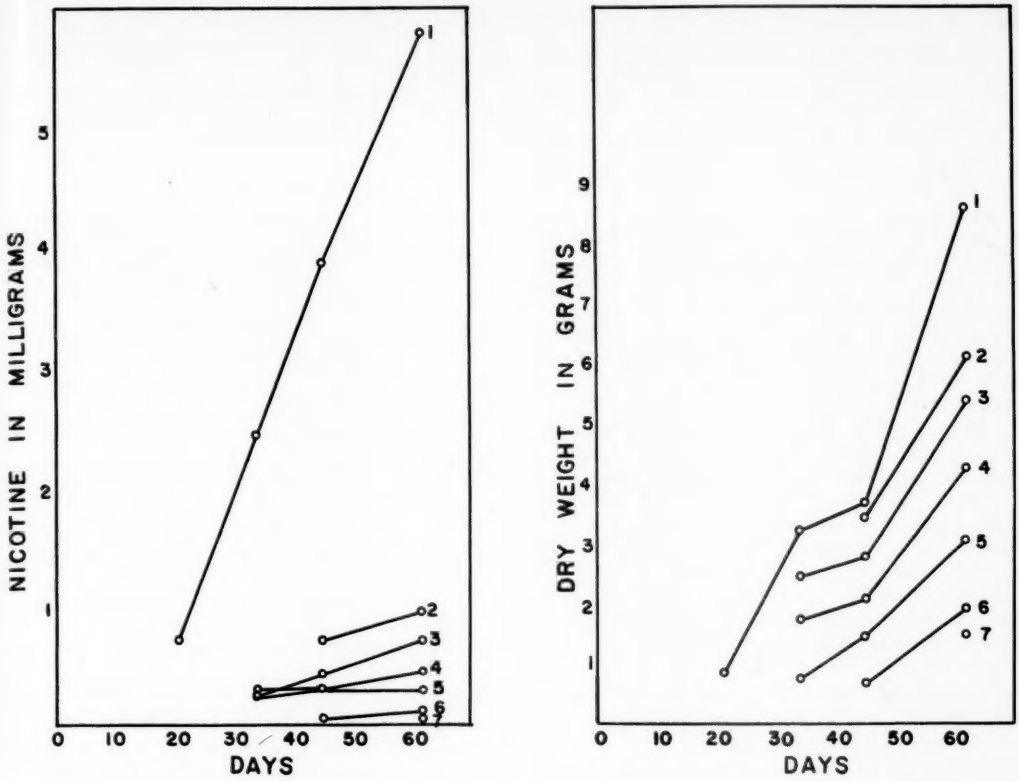
The changes that occurred in Connecticut 49 resemble those of Turkish with two exceptions. First,

Table VI

Nicotine, Dry Weight, and Length/Leaf of Leaves Taken from Scions of Nicotiana rustica var. brasilia Grown on Tomato Rootstocks

Leaf no. from base of scion	Nicotine, μgm				Dry wt, gm				Length, mm			
	24 Days	42	52	72	24	42	52	72	24	42	52	72
1	15	8	20	15	0.54	0.39	0.45	0.63	250	207	212	232
2	21	7	26	0	0.51	0.75	0.66	0.96	243	268	262	284
3	27	15	27	14	0.95	0.96	1.09	1.07	305	311	320	309
4	41	18	58	15	0.80	1.14	1.11	1.99	265	311	312	348
5	15	31	56	0	0.70	1.44	1.68	2.17	250	346	359	368
6	21	25	59	87	0.63	1.51	1.88	2.35	212	326	366	356
7	20	31	76	0	0.30	1.56	1.60	3.04	150	341	338	391
8	7	76	32	tr	0.21	1.68	2.11	2.49	111	357	364	358
9	11	26	108	0	0.12	1.49	2.11	3.59	99	337	363	378
10	..	25	38	0	...	1.56	2.09	3.06	...	336	361	361
11	..	27	37	0	...	1.40	2.40	2.61	...	309	369	347
12	..	21	93	0	...	0.99	1.82	3.06	...	248	330	339
13	..	18	0	0	...	0.76	1.87	2.22	...	215	323	299
14	..	9	18	0	...	0.62	1.84	2.16	...	188	311	295
15	..	8	17	0	...	0.41	1.49	2.02	...	145	268	273
16	..	10	10	0	...	0.25	1.36	1.49	...	117	241	239
17	..	9	0	0	...	0.20	0.86	1.14	...	75	188	199
18	..	..	22	0	...	...	0.74	0.68	...	...	159	164
19	..	..	0	0	...	...	0.36	0.49	...	...	106	153
20	..	..	0	0	...	...	0.24	0.30	...	...	79	115
21	..	..	0	0	...	...	0.19	0.26	...	...	62	103
22	..	..	..	0	...	...	...	0.18	...	...	...	92
23	..	..	..	0	...	...	...	0.19	...	...	...	81

Plants were harvested at 4 stages of growth and each figure represents an average of 4 leaves.



FIGS. 1 and 2. The nicotine and dry wt accumulation in stems of *N. Tabacum* var. Turkish grafted on tomato rootstocks and harvested at 4 different stages in development. Stems were divided into segments each 162 mm long. No. 1 designates the basal segment adjacent to the graft union.

the quantities of alkaloid involved were only about one-half those of Turkish. Second, the basal leaves suffered losses of alkaloid similar to senescent losses in ungrafted plants. *N. rustica* showed small increases during the first three harvests with the peak of alkaloid accumulation moving further up the stem with each crop. In the final crop there was a complete shift from accumulation to loss with only the basal leaves containing detectable alkaloid.

The pattern of alkaloid accumulation in stems was studied in Turkish tobacco (figs 1 and 2). Accumulation occurred in all segments (each 162 mm long) but the amount in the first segment adjacent to the graft union was far in excess of the total of that in the other segments. This runs contrary to the dry weight distribution which in each segment increased at an approximately uniform rate. As an example, at the last harvest, segment 3 contained 63 % as much dry matter as segment 1 but only 13 % as much nicotine. Connecticut 49 also showed a similar accumulation pattern in which the first segment contained the greatest quantity of alkaloid, approximately 10 times as much as succeeding segments, while there was no trace in the top.

DISCUSSION

In general it can be stated that the leaf accumulates nicotine as it grows. This accumulation can be modified in basal leaves by normal senescent losses (as in Connecticut 49), by translocation from the lower part of the stem (as in Turkish), or in leaves at all levels by virtually complete disappearance at the time of fruiting (as in *N. rustica*). Except in leaves on young plants which have not reached their full size there is no relation between nicotine content and either length or dry weight. There is an indication that some leaves increase their alkaloid content out of proportion to either dry weight or length. In addition leaves at the high stalk positions accumulate progressively smaller amounts of alkaloid regardless of the dry weight attained. There is no interpretation for these observations at the present time.

The stalk also accumulates nicotine as it grows, although in older plants the terminal portion of the stalk as well as the entire inflorescence may be alkaloid-free (as in Connecticut 49). As in the case of leaves, a complete disappearance of nicotine from the entire stalk may occur at time of fruiting (as in *N. rustica*). The exceptionally large increases in alka-

loid in the basal segment of the stalk (fig 1) compared to segments higher up may be an interaction with the graft union. Similarities between proliferation of cells of the stem callus culture and of the graft may in part explain this abnormal increase.

The findings of Wahl (13) lead one to question whether such accumulations in the tobacco scion are the result of actual synthesis or of translocation from the tomato plant. The evidence against translocation and in favor of synthesis is as follows. 1) There is a complete lack of relationship between the data contained in the two columns of table I. The concentration of alkaloid in the stock is in no way related to that found in the scion. There is also a wide range of alkaloid contents found in scions of different species grafted to identical rootstocks (table II). 2) The nicotine in the scion is always higher than that of the stock and in most cases this difference is more than ten-fold. An exception can be stated in the case of *N. rustica* where the alkaloid concentration drops to the range of the tomato and then almost completely disappears. This disappearance appears to be indicative of a nicotine-destroying mechanism within the *N. rustica* plant. 3) The synthesis of nicotine by stem callus cultures during initial passages is further evidence that cells of the aerial portions of tobacco are able to make alkaloids. 4) The final and most conclusive evidence is the appearance of radioactivity in the nicotine of cut scions fed tritium-labeled nicotinic acid.

Summary

The pattern of alkaloid synthesis in the tobacco shoot was studied using two varieties of *Nicotiana Tabacum* (Turkish and Connecticut Shade no. 49) and one of *N. rustica* var. *brasilia* grafted to Marglobe tomato rootstocks. Total pyridine alkaloid increased rapidly at first and then progressively more slowly in collections taken at four stages of growth (the last at the seed pod stage). The order of increasing rates of accumulation was: *N. rustica* < Connecticut 49 < Turkish. Analyses of individual leaves showed that they accumulate nicotine as they grow but the upper leaves accumulate less per unit of dry weight than do the lower leaves. Turkish and Connecticut 49 stems contain about twice as much alkaloid as the leaves; two-thirds of this was in the lowest stem segment. Conclusive evidence for actual nicotine synthesis in the shoot was obtained by the isolation of radioactive nicotine from scions which had been excised and fed through their cut ends with tritium-labeled nicotinic acid.

The advice and encouragement of Professor R. F. Dawson of Columbia University has been greatly appreciated.

Tritium-labeled nicotinic acid was prepared by Dr. D. R. Christman of Brookhaven National Laboratory.

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BRIEF PAPERS

COMMENTS ON THE ABSORPTION OF INORGANIC IONS BY ROOT CELLS[1]

ROY OVERSTREET

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The findings of modern microscopy reveal a seemingly unlimited physical organization in living protoplasm. At the same time, they point up the extreme lability of many protoplasmic structures. For example, the endoplasmic reticulum, a continuous network of membrane-bound cavities that permeates the cytoplasm of animal cells, breaks down on injury or cytolysis into a collection of isolated vesicles (10). Also, it is well known that structures such as the mitochondria may be profoundly altered as a result of cellular changes or variations in experimental conditions (1).

In terms of organic chemistry, the structures of the protoplast are equally elaborate. From even a cursory survey of plant biochemistry one is impressed by the extreme complexity of living structures and by their instability or transitory nature.

In the present communication, the writer wishes to point out that much of the puzzling information concerning the absorption of inorganic ions by plant tissues becomes more comprehensible when viewed in terms of protoplasmic structures. That is, the mineral absorption and isotopic exchange data regarding root tissues suggest that inorganic ions may be laid down as component units of highly complex and unstable chemical arrangements in cells. Certain of the ideas to be presented are similar to those put forth a decade ago by Steinbach on the basis of the behavior of inorganic ions in striated muscle cells (11). A few examples of the mineral absorption data will serve to present the general concept.

It has been commonly observed that many plant tissues lose absorbed ions such as K^+ to dilute solutions of HCl. With barley root tissue, essentially all of the absorbed K^+ is released in a two-hour period to HCl solutions of normality 10^{-3} or greater (7). A further significant aspect of the K^+ release is the concomitant and proportionate loss of a variety of other cellular constituents such as inorganic phosphorus, soluble organic phosphorus, soluble nitrogen, and Ca^{++} (7). That is, hydrogen ion concentrations that induce the release of a particular absorbed ion also effect a release of all other absorbed cations and anions as well as certain organic components of the cell. Conceivably, this experimental observation could be explained as the result of an effect of hydrogen ion on the properties of certain cytoplasmic membranes. In the writer's opinion, it could be equally well explained on the basis of an instability to H^+ of a chemical structure in which all of the absorbed ions are represented.

The loss of K^+, Ca^{++}, and other cellular constituents to dilute HCl solutions can be offset completely or in part by the addition of K^+ or Ca^{++} to the acid media (7). This is perhaps analogous to certain phenomena of simple inorganic chemistry. For example, the compound $CaK_2(SO_4)_2 \cdot H_2O$ dissolves readily and is unstable in acid solutions because of the formation of HSO_4^- ion. However, in conformity with the laws of physical chemistry, the salt dissolves less readily in and is more stable with respect to acid solutions containing Ca^{++}, K^+, or $SO_4^=$ ions.

From rate studies of isotopic exchange between excised root tissue and the culture medium, it must be concluded that K^+ ion is much more firmly bound within the tissue when it is absorbed along with Ca^{++} ion (9). To the writer this experimental fact suggests that both K^+ and Ca^{++} are laid down in a single chemical structure and this structure is more stable than that formed when K^+ is absorbed in the absence of Ca^{++} ion.

A similar hypothesis might be invoked to explain the marked stimulating effect that Ca^{++} ion can have on the absorption rates for K^+ and Br^- ions (12). That is, when absorbed along with Ca^{++}, K^+ and Br^- ions are incorporated in a more stable structure and this fact is reflected in enhanced absorption rates for these ions.

However, since a number of polyvalent ions such as Al^{+++}, Ce^{+++} and La^{+++} also have stimulating effects on the absorption of K^+ (4), it appears necessary to envisage a series of possible chemical structures within the protoplast of varying stability. In this series the amounts of component cations and anions can vary, subject of course to the overall restriction of electric neutrality of the unit. Instances of situations of this kind are common in crystal chemistry. For example, a series of alumino-silicates possessing the mica structure is known in which the proportions of Al^{+++}, Mg^{++}, Fe^{++} and K^+ can vary widely.

For a given plant tissue and nutrient environment, it appears reasonable that cations and anions will be absorbed in such proportions and amounts as to form the most stable structure. Thus on this picture, competitions between ions of like sign and mutual effects between ions of unlike sign in absorption are to be expected. Specifically, this hypothesis allows for the experimental observation that K^+ is less rapidly absorbed when Na^+ is present or that K^+ is more rapidly absorbed from a KCl solution than from a K_2SO_4 solution of the same equivalent concentration.

On the basis of the structural concept, one would

[1] Received May 7, 1957.

further predict that when tissues are transferred from one nutrient environment to another, readjustments may occur involving the breaking down of old structures and the building up of new structures appropriate to the new environment. Also, when tissue is transferred from a relatively concentrated nutrient medium to a more dilute medium or to distilled water, the readjustments may involve releases of absorbed cations and anions to the culture solution. It has been observed in this laboratory that when barley root tissue which has been grown in a medium high in KBr is transferred to distilled water, important fractions of the total absorbed K^+ and Br^- are readily released. A similar line of reasoning may serve to explain the presumed reversible diffusion of salts into the protoplasm of many cells upon which calculations of the socalled "apparent free space" are based (2, 5, 6).

Among recent workers in the field of mineral absorption by cells, the postulation of metabolically produced "carrier substances" is general. By carrier substances are understood organic compounds capable of forming complexes with or sequestering inorganic ions. According to some views the carriers possess discrete chemical structures such as can be attributed to enzymes (3). A theoretically necessary part of all accumulation models involving carriers is a barrier or membrane which is permeable to the carrier-ion complex but which is relatively impermeable to free ions (8). Thus at the outer boundary of the barrier, the ions combine with the carriers and the resulting complexes diffuse through the barrier. On the inner surface of the barrier, the complexes are broken down chemically and the ions are released.

The reasoning presented in this communication constitutes a modification of the usual concept of the carrier substance and, as regards the mechanism of ion accumulation, places more emphasis on structure than on membranes and limiting boundaries with special chemical and physical properties. On this approach, the carrier is the living protoplasm itself in which the nutrient cations and anions are pictured as not being free but held as integral parts of a highly complicated and labile chemical structure. This means that the carrier substance and the barrier are one and the same thing. Also, the carrier substance is the same for both cations and anions. Finally, the carrier is not a discrete chemical compound but may vary as to its ionic content, and perhaps in other respects, according to experimental conditions.

On this picture, the formation of vacuoles and the secretion of ions to vacuoles are secondary effects which correspond to a breakdown, perhaps as a result of enzymatic action, of protoplasmic structure.

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EFFECTS OF GIBBERELLIC ACID ON THE PHOTOPERIOD-CONTROLLED GROWTH OF WOODY PLANTS[1,2]

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The discovery that gibberellic acid can replace the long day requirement for flowering in certain species of long day plants (2, 5, 6, 10), has suggested the further question of whether the long day requirement for vegetative growth characteristic of many species of woody plants (1, 4, 9) might also be replaced by applications of gibberellic acid. Lona has reported that cuttings of *Fagus sylvatica* maintained in a dormant condition by short day resume growth as a re-

[1] Received May 16, 1957.

[2] Report of work supported in part by the Herman Frasch Foundation.

sult of gibberellic acid treatment (8), and Cooper has found that gibberellic acid treatment of *Citrus paradisi* (grapefruit) growing in the Rio Grande valley, in December, may cause renewed vegetative growth in these trees (3).

The present paper will report the initiation of vegetative growth in Camellia under short day conditions, as a result of the periodic application of gibberellic acid; and the failure of two other species to respond under similar conditions.

Three species of woody plants: *Camellia japonica*, var. Finlandia (Camellia), *Pinus Coulteri* (Coulter pine); and *Pseudotsuga macrocarpa*. (big cone spruce), all known to have a long day requirement for the initiation of vegetative growth, were selected for the present work. None of these species appears to possess a requirement for low temperature treatment to break physiological dormancy.

Dormant, two-year-old potted plants of each species were obtained from nurseries in which they were growing under field conditions (in the vicinity of Pasadena) and were transferred to the greenhouse in November. All plants were maintained on short (9-hr) days for one week and were then distributed among day length and temperature treatments. In one treatment, plants were maintained on long (20-hr) days and at a minimum night temperature of 18° C. In a second treatment, plants were maintained under short (9-hr) days and at a minimum temperature of 18° C. In the third treatment 8-hour days were combined with low (5° C) night temperature. Twenty plants of each species were used for each daylength and temperature treatment. Of these, half were treated with gibberellic acid. Short days were maintained by covering the plants every evening at 4:30 PM with a light-tight cloth and uncovering them every morning at 7:30 AM. Long days were obtained by extending the natural daylength to 20 hours with incandescent lights. Low temperature dark periods were achieved by placing the plants each evening in a household type electric refrigerator and returning them to the greenhouse each morning. The greenhouse was maintained at a minimum temperature of 18° C and occasionally reached a maximum temperature as high as 30° C. Gibberellic acid was applied to the terminal bud of each plant as a 0.004 ml alcoholic drop, giving a dose of 4 or 20 μgm of gibberellic acid per plant, as indicated below.

Treatment was started November 29 with the application of 4 μgm of gibberellic acid to each treated plant and the treatment was repeated one and 2 weeks later. The treatments were then increased to twice weekly until January 21, when they were again increased, to 3 times weekly. On January 30, the dose was increased to 20 μgm per plant (by increasing the concentration) and the thrice weekly applications were continued until the termination of the experiment.

By January 10 (about 6 weeks after transfer from short day), the Camellia plants on long day began to break dormancy and to resume vegetative growth. The plants treated with gibberellic acid commenced growth noticeably earlier than the control plants. Four to 6 weeks later most of the gibberellic acid treated plants on the short day, high night temperature regime, began to grow. Still later, several of the non-gibberellic acid treated short day control plants also broke dormancy. This variety of Camellia (Finlandia) shows therefore a less complete control of growth by daylength than the seedlings reported on earlier (1) which remained completely dormant for 1 year under short day. At the end of the experiment (April 20) 9 out of the 10 gibberellic acid treated plants on short day had resumed growth, while 4 of the 10 control plants had also broken dormancy. Non-gibberellic acid treated Camellia plants which received low night temperature in addition to short day remained completely dormant throughout the entire course of the experiment. Under this condition, too, however, treatment with gibberellic acid caused all of the treated plants to break dormancy and resume growth.

Plants maintained on long day formed a new terminal bud and ceased their growth after 6 to 8 weeks. This was true of both the gibberellic acid treated plants and the control plants. The gibberellic acid treated plants grew little more than the controls.

The extent of the experimentally induced growth (current growth) as well as the length of the stems at the beginning of the experiment (old growth) is shown in table I. At this time (April 20) the long day plants had ceased growth and formed terminal buds, and the short day gibberellic acid treated plants also appeared to have ceased growth, although the terminal buds were still small.

Within 6 to 8 weeks exposure to long days both Pinus and Pseudotsuga resumed vegetative growth. No significant effect of gibberellic acid was apparent, either in promoting a more rapid response or causing

TABLE I

AVERAGE LENGTH OF OLD AND CURRENT GROWTH ON THE MAIN STEM OF CAMELLIA JAPONICA VAR. FINLANDIA IN RESPONSE TO PHOTOPERIOD, NIGHT TEMPERATURE AND GIBBERELLIC ACID APPLICATIONS (DURATION OF THE EXPT—5 MOS)

GROWING CONDITIONS	GIBBERELLIC ACID TREATMENT	OLD GROWTH, CM	CURRENT GROWTH, CM
Long days—			
High night	–	27.1 ± 1.05	5.6 ± 0.67 *
temperature	+	25.6 ± 1.06	7.7 ± 0.42
Short days—			
High night	–	28.5 ± 1.04	1.7 ± 0.75
temperature	+	29.8 ± 0.87	5.9 ± 1.16
Short days—			
Low night	–	28.7 ± 0.89	...
temperature	+	28.4 ± 0.75	5.8 ± 1.05

* $\sqrt{\dfrac{\Sigma x^2 - \frac{1}{n}(\Sigma x)^2}{n(n-1)}}$.

a greater rate of growth. After about 4 months, 10 to 20% of the plants on short day began to show the initial indications of resumed growth, but gibberellic acid was again without apparent effect.

Dormancy, induced and maintained by short day conditions, may be very effectively broken in the Camellia by the periodic application of gibberellic acid. However, gibberellic acid applications were found to have no effect on dormancy in two other species, Pinus and Pseudotsuga. The response of dormant plants which require long days for vegetative growth is, then, similar to the response of species which require long days for flowering, in that gibberellic acid is fully effective in replacing long day in some species, but is apparently completely ineffective in others (7).

The dormancy of these evergreen species is environmentally controlled. Dormancy, in the sense of cessation of growth, may be induced by transfer of the plant to short days. If the plant is then returned to an environment favorable for growth (long, warm days), growth is resumed. Plants maintained on continuous long days grow in flushes. After a period of growth the plant becomes dormant again. This is ultimately followed by a second period of growth, after which the entire process may be repeated.

The cessation of growth under favorable environmental conditions appears to be some form of physiological dormancy. Nothing is known as yet about the causes of this dormancy or how it may be modified. Gibberellic acid does not prevent it onset. When the buds are physiologically ready to resume growth, gibberellic acid can replace the long day environmental treatment usually required to initiate new growth.

The Camellia plants used in this study (2-yr-old rooted cuttings of the variety Finlandia) were kindly supplied by Mr. Julius Nuccio, Nuccio Nurseries. The seedling plants of *Pinus Coulteri* were kindly supplied by Mr. Gus Juhren, U. S. Forest Service. The seedling plants of *Pseudotsuga macrocarpa* were kindly supplied by Mr. R. M. VanWagener, Los Angeles County Forester and Fire Warden Department. The authors also wish to thank Dr. Henry Hellmers for his help and counsel.

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THE RELATIONSHIP OF TEMPERATURE AND NUTRITION TO THE GROWTH OF SUNFLOWER TUMOR TISSUE IN VITRO[1,2]

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For the past fifty years the effects of temperature on plant and animal tissue cultures have received the attention of relatively few investigators.

In the area of plant research most of the heat studies have been with in vivo material. The role of temperature in plant tumor formation has received some attention. Riker (6) found that the development of crown galls on tomato plants varied with the surrounding temperatures of both soil and air. Soil temperature of 22° C seemed to be best for crown gall development on stems while development was poor at air temperatures of 28 to 30° C, with no development above 30° C. These results were confirmed and further expanded by Braun (1). De Ropp (2) demonstrated that tumor formation on sunflower stem sections grown in vitro is inhibited entirely when cultured at 33° C with no apparent effect on the stem fragments (normal tissue) nor the bacteria. This in-

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[2] This investigation was aided by Research Grant C-1632 from the National Cancer Institute of the National Institutes of Health, U. S. Public Health Service.
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dicates that the postulated tumor inducing principle (TIP) (5) is either heat labile or is inactivated by some cellular constituent at 33° C.

The work reported here will show the varying effects of elevated temperature on sunflower tumor tissue grown on different media. Comparable observations, however, have at the same time been made on habituated tissue. A probable explanation is given for the growth modification due to abnormally high temperatures.

The sunflower tumor (P_v) and habituated tissues (H_h) used in these experiments had been maintained in continuous culture on four media at 25° ± 1° C for several 6-week culture periods. These media were composed of salts and sugar only (GB); salts and sugar with the addition of casein hydrolysate, adenine sulfate and a mixture of vitamins (GBA). Both were used with and without IAA at 0.05 mg per liter (3). The initial inocula averaged 100 mg wet wt. An original exposure of these tissues (tumor and habituated) to high temperature was made accidentally. This resulted from mechanical failure in the cooling system of the incubator room and for five weeks while it was out of repair the incubator room temperature reached as high as 33 to 34° C. A significant decrease in weights of the tumor tissue was noted following the period of elevated temperature.

The effects of thermal stress on tumor tissue as indicated by the weight decline were noteworthy (fig 1). All tissues during the second transfer period were affected by the abnormal temperature as indicated by a sharp decrease in weights. Tissues on the medium GB – IAA show a continuous loss in weight over the subsequent culture periods, but those on the medium GB + IAA did overcome the stress of heating after about four transfers and returned to a normal pattern of growth. The tissues grown on GBA with or without IAA did not exhibit, over several transfer periods, a definitive pattern in weight decline, rather there was a cyclic fluctuation in their weight values. It is probable that the abnormal temperatures had little or no influence on these tissues. Habituated tissues growing on the complete medium, GBA + IAA, were likewise unaffected (fig 2).

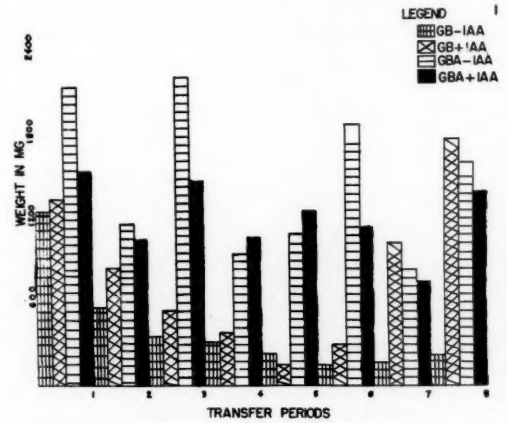

FIG. 1. The effect of exposure of sunflower tumor tissue (P_v) to abnormal temperatures (33 to 34° C) over a 5-wk period (Transfer Period 2), followed by a controlled temperature of 25° C. Transfer periods were at 6-wk intervals.

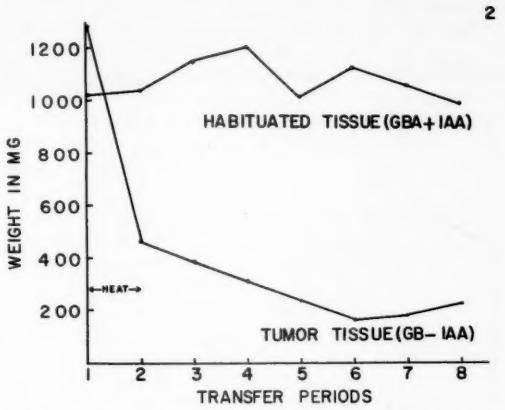

FIG. 2. A comparison of sunflower tumor and habituated tissues grown on their respective media during and after the thermal stress.

When a comparison is made between tumor and habituated tissues grown on their respective standard media (3), i.e., the former on GB – IAA and the latter on GBA + IAA, there is a similarity in the growth rate of these two tissues at 25° C (fig 2; see also (4), table 5). Following the abnormal temperature period referred to above, however, the rate of growth declined for the tumor tissue, whereas there was no change in the rate of growth of the habituated tissue (fig 2). These results would indicate that the higher temperature, rather than any of the components of the medium, must have an effect on the tumor tissue itself, and that there is not a similar influence on the habituated tissue in the presence, at least, of the complete medium.

Explants of the poorly growing tumor tissue were put on the complete medium. At the end of the usual culture period of six weeks, their average weight was increased threefold. For the ensuing culture periods these tissues were divided into two lots. One lot was continued on the complete medium (GBA + IAA) while the other was returned to the basal medium (GB – IAA). Those on the complete medium continued to grow well, while the tissues that were on the old (basal) medium grew poorly (fig 3).

The normal continual growth and development of sunflower tumor tissue can be altered upon exposure to high temperature. The limited initiation and formation of tumors on both the whole plant (6) and plant parts, i.e., the stem (2) when under thermal stress, has been demonstrated. Also, in most instances the change in tumor tissue responses is ac-

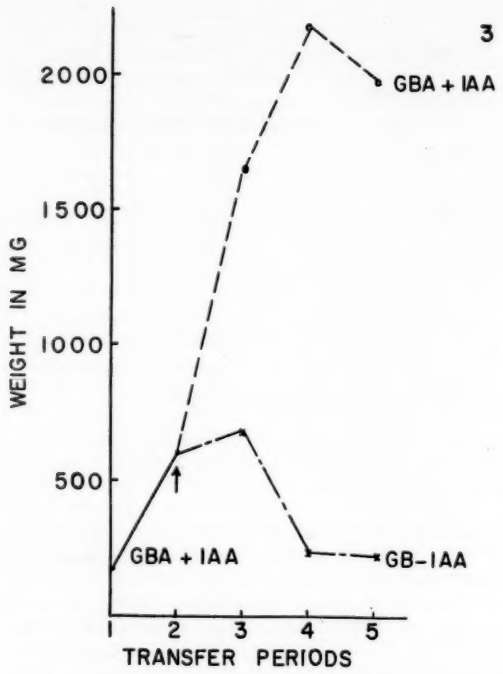

FIG. 3. The influence of complete medium on tumor tissue after thermal treatment. The tissues were placed on complete medium (GBA + IAA) for one period after the heated condition and then divided into two groups (arrow), one continuing on complete medium, the other on the basal medium (GB - IAA).

companied by no change in the surrounding normal tissue under the same environmental conditions in vivo.

According to Braun (1), at 32° C the tumor inducing principle, TIP, is altered and inoculated cells when held at this temperature fail to become tumorous. Although several postulations have been made concerning the nature of TIP, it remains to be fully understood (5).

The findings in this report suggest that probably the TIP and the principle responsible for continuous growth and development in vitro, "tumor progression principle", TPP, are similar in nature. This can be substantiated in part by the fact that the TIP and TPP responses to thermal stress are similar, and that within the same temperature range they may be either retarded or destroyed.

The interrelationship of nutrition and temperature with TPP is significant. Because of the nature of sunflower tumor tissue, it can be maintained on a very simple medium consisting of salts (nitrates, etc.) and sugar (usually sucrose). However, for the continuous culture of normal tissue in vitro several other substances must be present in the medium. In addition to the salts and sugar a supplemental mixture of vitamins, amino acids, a purine base, and auxin are supplied, if continuous growth is to be obtained (3). The addition of these supplements to sunflower tumor tissue medium will also increase growth of tumor tissue, although these are not necessary for its continuous maintenance. Tumor tissue either does not need these supplemental nutrients or it is able to synthesize those which are required.

The idea that tumor tissue may synthesize certain substances required for growth may be supported in part by its growth response when exposed to heat. Tumor tissue, grown on the basal medium minus supplements, at high temperature loses much of its capacity to grow. After the thermal stress the tumor tissue response is similar to that of habituated tissue grown on the basal medium minus supplements and auxin (fig 4). When this tissue is placed on the complete medium, however, the rate of growth is increased, which indicates the apparent need for a substance, the synthesis of which is impaired by heating. Since the medium is sterilized by autoclaving, it is reasonable to expect that a temperature of 30 to 34° C could have no further effect upon the components of the medium. Therefore, any change in tumor growth response caused by the thermal stress is due to some alteration within or in the presence of the tissue. It is reasonable to suspect that the underlying cause may involve one of several factors: destruction or inactivation of a key component, influence on an enzyme system, or an alteration in the general metabolic pattern of the tissue.

Assuming an active TPP to be necessary for the normal growth of tumor cells, it would be logical to conclude that the role of TPP could be the factor affected if the heat treatment resulted in less or retarded growth. This speculation is substantiated by the evidence shown in figure 1. The influence of TPP might possibly be effective at some stage prior to the formation of amino acids. The addition of supplements, including amino acids, overcomes this de-

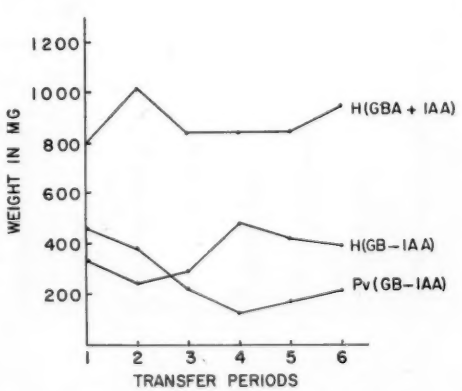

FIG. 4. The similarity between habituated tissue and heat-treated tumor tissue grown on the basal medium, compared with habituated tissue on complete medium.

pressed growth. It appears from the evidence in figure 3 that after exposure to heat the TPP is not restored or reformed by this addition, but that for the accelerated growth the supplements must continually be added to the medium. Since the role of TPP is partially replaced by the supplements, it seems plausible that the role of TPP is involved in the normal supply or synthesis of the required components present in the supplement.

When IAA is in the medium, during and after thermal stress, the tissue will slowly overcome the deficit in growth and return to the growth pattern exhibited prior to heating. IAA then appears to be a part of the causative factor responsible for the autonomous nature of tumor tissue. The TPP may be formed as a result of the influence or presence of IAA with certain other substances which are easily degraded by heat or whose synthesis is impaired by heat. These substances are ordinarily synthesized by tumor tissue along with auxin. If this synthesis is altered or the substances destroyed, tissue growth is retarded.

Summary

The growth of sunflower tumor and habituated tissues on several media was observed. Tumor tissue growth was found to be more retarded at 33 to 34° C on medium lacking supplements, than when they were grown at this same temperature on medium containing supplements. The autonomous nature of tumor tissue is altered at temperatures above the normal for growth. The presence of supplements in the medium offers a partial replacement for a naturally occurring factor (TPP) in tumor tissue which is wholly or partly responsible for its autonomous nature. IAA is essential to the recovery of growth of the tissue.

Habituated tissues are unaffected by the temperature change when grown on the complete medium, GBA + IAA.

The authors wish to acknowledge the suggestions and advice of Dr. R. H. Wetmore and Dr. K. V. Thimann in the preparation of the manuscript.

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THE KINETICS OF POTASSIUM ACCUMULATION BY CORN ROOTS AS A FUNCTION OF CELL MATURITY[1]

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Brown and Cartwright (1), using serial sections of corn roots, have reported that as cells vacuolate and mature, their capacity to accumulate potassium on a cell or protein basis increases. This finding raises the question as to what changes have occurred during growth that enable the cell to be more effective in ion accumulation. Brown and Cartwright have suggested that vacuolation is important, in that it introduces the tonoplast and provides a depository for absorbed ions. However, they also note that once the vacuole is established the increase must be partially attributed to a differentiation of the cytoplasm such that the absorption per unit protein increases. This last observation is of considerable importance in the study of the carrier mechanism of potassium accumulation, for the mature cells must possess a more extensive or more efficient carrier mechanism than immature cells.

In order to obtain further information about these changes in ion accumulation with cell maturation, we have investigated the kinetics of potassium accumulation in serial corn root sections representing different mean levels of cell maturity. The techniques and solutions were those described in a previous report (2). Vigorous roots from corn seedlings germinated in the dark for 4 days were cut into a 5-mm tip section (including the root cap) and 3 subsequent-10 mm sections. Treatments were carried out in quadruplicate, with 10 sections per flask shaken for 3 hours in 15 ml of a KCl-KH_2PO_4 solution (pH 5.6), labeled with Rb^{86}. Exchangeable ions were removed and accumulated ions determined from the radioactivity of the ashed sections. The effect of calcium was determined by making the uptake solutions 0.0005 N with respect to $CaCl_2$. Kinetic constants were calculated as described in the previous report (2). Protein nitrogen content of the sections was determined by homogenizing in 10 % trichloroacetic acid, and analyzing the centrifuged precipitate by digestion and Nesslerization. The data are presented in table I.

The constant K_s, which closely approximates the dissociation constant of the carrier-ion complex (2), varies with the maturity of the root tissue. In the

[1] Received May 28, 1957.

TABLE I

KINETIC CONSTANTS OF POTASSIUM ACCUMULATION BY SERIAL CORN ROOT SECTIONS *

ROOT SECTION, MM FROM TIP	K_m, MEQ/L		V_{max}, μEQ K × HR^{-1} × G M FRESH WT^{-1}		× MG PROTEIN N^{-1}	
	− Ca	+ Ca	− Ca	+ Ca	− Ca	+ Ca
0– 5	0.34	0.89	8.73	3.01	1.48	0.51
5–15	0.44	0.44	2.36	0.77	3.60	1.18
15–25	0.47	0.32	1.63	0.71	3.17	1.38
25–35	0.55	0.11	1.77	1.01	2.74	1.57
Entire root	0.51	0.19	2.50	1.99	...	...

* Mean values from 4 expts. Values for entire primary roots were taken from a previous report (2) and inserted here for purpose of comparison.

absence of calcium it rises with increasing cell maturity; conversely, in the presence of calcium it falls. In the tip section calcium decreases the affinity between potassium and its carrier; in the more basal sections the affinity is increased.

On a fresh weight basis, V_{max}—the maximum velocity at which the tissue can accumulate potassium under these conditions—is maximal in the tip region and minimal in the 15 to 25 mm region. On a protein nitrogen basis, V_{max} is maximal in the 5 to 15 mm region in the absence of calcium and in the 25 to 35 mm region in its presence. Both with and without calcium the minimum velocity occurs in the root tip. Calcium depresses V_{max} less in the basal sections than in the tip section. Although these sections are larger and encompass a greater root length than those used by Brown and Cartwright, the data verify their observation that on a protein basis the immature cells are not as effective in potassium accumulation.

The changes in the kinetic constants and in their response to calcium with cell maturation can be explained in various ways. A number of carrier systems can be operative in potassium accumulation, each with different characteristics, the measured kinetic values being but the mean of the contribution to accumulation made by each carrier. During the ontogeny of the root cell the proportion or activity of the several carriers may change—and perhaps new carriers are introduced—with a resultant alteration of the constants of accumulation. On the other hand, a single carrier system with sequential steps may be altered in its biochemical and biophysical properties by the alteration of one or more of the steps. Kinetic studies are of limited value in deciding between these or other alternatives as they give only the overall characteristics of the individual reactions involved, and are insufficient to determine the complexity and diversity of the individual steps. We can only guess as to the steps occurring between the initial and the final phase, with the sole knowledge that the complex reaction has to obey the Michaelis-Menten kinetics. A similar difficulty involving the adenosine triphosphate activation of myosin has been reviewed by Morales, Botts, Blum and Hill (3).

SUMMARY

The kinetics of potassium accumulation by serial sections from corn root tips have been investigated. The kinetic constants, K_m and V_m, change with growth and maturation, and the nature and extent of the change is conditioned by the presence or absence of calcium ion. The velocity of potassium accumulation per unit protein nitrogen is least in the meristematic region. It is deduced that K is accumulated by a complex of carriers operating independently or sequentially, and that elements of the complex are changed during growth and maturation.

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OXIDATION OF MALONATE BY PEANUT MITOCHONDRIA [1]

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For many years malonate was considered a classical competitive inhibitor of succinic dehydrogenase (1). The assumption was made that it is a metabolically inert substance. However, the utilization of malonate by microorganisms (2–6), mammals (7–9), and fishes (10) suggests that this acid may play a role in organic acid metabolism. Studies with a partially purified enzyme obtained from *Pseudomonas* sp. (6) and mitochondria from rat kidney (9) suggest that malonate degradation proceeds by way of malonyl CoA, which is then decarboxylated to acetyl CoA and CO_2.

Malonate has been reported in the leaves of a

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TABLE I

COFACTOR REQUIREMENTS FOR OXIDATION OF MALONATE-1,3-C^{14}

COMPONENTS	% OXIDATION
Complete	24
- ATP	2
- CoA	2
- DPN	14
- TPN	16
- GSH	17
- $MnSO_4$	11

The complete reaction mixture contained 1 micromole of malonate-1,3-C^{14}, 5800 cpm, 0.5 ml of mitochondria (approximately 22 mg of protein) in 0.2 M TRIS-0.5 M sucrose, pH 7.2, with about 5×10^{-3} BAL, 10 micromoles of phosphate buffer, pH 7.1, 50 micromoles of KCl, 1 micromole of ATP, 0.3 micromole of CoA, 0.2 micromole of DPN, 0.1 micromole of TPN, 5 micromoles of GSH, 1 micromole of αKG, 1 micromole of $MnSO_4$, 0.2 ml of 20% KOH in the center well, 0.3 ml of 10 M H_2SO_4 in the sidearm, final volume 1.7 ml. Time of incubation, 2 hrs; temperature, 25° C; gas phase, air; % oxidation = $BaC^{14}O_3$ (cpm) × 100/substrate (cpm).

number of plants (11). However, the unequivocal utilization of malonate by plant tissues has not been demonstrated (12).

In the course of an investigation on the oxidation of propionate to CO_2 and acetate by peanut mitochondria (13) we observed a rapid release of $C^{14}O_2$ from 1 micromole of malonate-1,3-C^{14} (table I). An absolute requirement for ATP[4] and CoA was demonstrated. DPN, TPN, GSH, Mn^{++} and α-ketoglutarate enhance the rate of $C^{14}O_2$ released. While 1 micromole of malonate-1,3-C^{14} is oxidized 24% in 2 hours, malonate-2-C^{14} under the same conditions is oxidized only 4.5%. This would be expected if malonate is oxidized via acetyl CoA which then enters the Krebs cycle.

The path of malonate oxidation was determined by incubating malonate-2-C^{14} with peanut mitochondria in the presence of all the cofactors shown in table I. The reaction products were examined for Krebs cycle acids by standard paper chromatographic techniques.

[4] The following abbreviations are used: adenosine triphosphate, ATP; coenzyme A, CoA; diphosphopyridine nucleotide, DPN; triphosphopyridine nucleotide, TPN; glutathione, GSH; α-ketoglutarate, αKG; 2,3-dimercaptopropanol, BAL; 2-amino-2-hydroxymethyl-1,3-propanediol, TRIS.

When 0.1 micromole of malonate-2-C^{14} was oxidized, the Krebs cycle acids, citrate, malate and succinate became radioactive. It would appear from this evidence that at low concentrations, malonate is readily metabolized by the following scheme:

$$\text{malonate} \xrightarrow{\text{ATP, CoA}} \text{malonyl CoA} \rightarrow CO_2 + \text{acetyl CoA} \rightarrow \text{Krebs cycle}$$

The details of this sequence are now under investigation.

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EFFECT OF SOME ISOMERIC PURINE ANALOGUES ON GERMINATION OF LETTUCE SEED[1]

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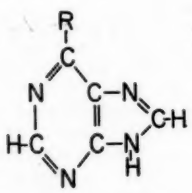
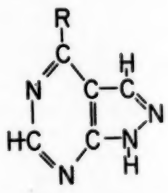
The stimulation of lettuce seed germination in the dark by certain 6-(substituted)purine derivatives has recently been demonstrated. The activity of these compounds, in this respect, is not restricted to kinetin (6-(2-furfuryl)aminopurine) alone (1); but many other 6-(substituted)aminopurines have been shown to have activity equal to, and in some cases, greater than kinetin (3). Further, the presence of a substituted amino group in the 6 position of the purine nucleus does not appear to be necessary, since analogous 6-(substituted)thiopurines have also been observed to have this biological effect of stimulating lettuce seed germination in the dark at high temperatures (2).

In an effort to examine the extent of chemical specificity necessary to retain activity in this type of compound, a number of substituted pyrazolo (3,4-d)-pyrimidines[2] were tested under the experimental conditions previously used to study analogous purine compounds. The difference in chemical structure within this series, as compared to the purine derivatives, is a change in the position of the nitrogen atom in the 5-membered ring portion of the heterocyclic moiety, e.g.,



6-(SUBSTITUTED)PURINE



4-(SUBSTITUTED)PYRAZOLO-(3,4-d)PYRIMIDINE

There were available for testing eight analogues with the same R side chain, but with the different heterocyclic nuclei; and in five other examples there were available additional members in the pyrazolo (3,4-d)pyrimidine series which were assayed for their effects on stimulation of lettuce seed germination.

In addition to the above mentioned pyrazolo(3,4-d)pyrimidine derivative some 13 additional purine derivatives were tested which possessed various substituted groups in one or more positions.

The biological tests were made by a previously reported procedure (3) in which lettuce seed (Early Curled Simpson) were pre-soaked in a given concentration of the compound to be tested for 8 hours in the dark at 25° C. The seeds were drained, and placed on filter paper, wet with the corresponding chemical solution, in petri dishes, and allowed to germinate in the dark at 30° C. All of the mechanical operations were carried out in the presence of a blue light (fluorescent light filtered through four layers of duPont 300 MSC dark blue cellophane). For a routine assay, the seeds were counted after 48 hours; and at least 100 seeds were used for each test.

For comparative purposes, activity in the stimulation of seed germination by some 6-(substituted)aminopurines are compared to the isomeric analogues in the pyrazolo(3,4-d)pyrimidine series (table I). It was observed that none of the latter derivatives stimulated germination, even though the identical side chain was present, and in the same relative position in pyrimidine portion of the molecule. In three examples, furfurylamino-, benzylamino-, and *n*-butylamino, the analogous purine derivative stimulated germination up to 80 to 90 % as contrasted to a water control reading of about 3 to 6 %; whereas the corresponding isomeric purine analogues possessed essentially no activity. That these latter compounds were not toxic was shown by the fact that seeds pre-treated

TABLE I

SEED GERMINATION ACTIVITY OF SOME ANALOGOUS SUBSTITUTED PURINE AND PYRAZOLO(3,4-d)PYRIMIDINE DERIVATIVES

CONC OF PRE-SOAK SOLUTION	SUBSTITUTED GROUP	4-(SUBSTITUTED)-PYRAZOLO(3,4-d)-PYRIMIDINE *	6-(SUBSTITUTED)-PURINE
μgm/ml		*% seed germination in 48 hrs* **	
3	Benzylamino-	7	
10		4	87
30		4	
3	(2-Furfuryl)amino-	9	
10		4	81
30		2	
3	*n*-Butylamino-	3	
10		2	86
30		10	

* Other compounds which were tested and found to be relatively inactive at the same concentrations include: 4-mercapto-, 4-amino-, 4-methylamino-, 4-ethylamino-, 4-dimethylamino-, 4-methylthio-, 4-amino-1-methyl-, 4-amino-1-*p*-tolyl-, 4-isopropylamino-, 4-amipno-1-β-hydroxyethyl- and 4-methylamino-1-methyl-pyrazolo(3,4-d)pyrimidine.

** Control seed soaked in water alone averaged 3 to 7 % germination.

[1] Received June 21, 1957.

[2] These compounds were synthesized and kindly furnished to the authors by Dr. R. K. Robins, New Mexico Highlands University, Las Vegas, New Mexico.

TABLE II

SEED GERMINATION ACTIVITY OF SOME 6-(R)-9-(R')-PURINES

R	R'	SEED PRE-SOAKED IN SOLUTIONS CONTAINING *		
		3 μGM/ML	10 μGM/ML	30 μGM/ML
		% germination after 48 hrs		
Dimethylamino-	Methyl-	11	6	22
Methoxy-	Methyl-	12	16	12
Hydrazino-	-H	11	10	8
o-Chlorophenyl-amino-	-H	48	57	31
m-Chlorophenyl-amino-	-H	58	50	52
p-Chlorophenyl-amino-	-H	59	42	36
N-Methylphenyl-amino-	-H	36	48	51
3-Isopropoxy-propylamino-	-H	54	47	30
Chloro-	Methyl-	4	8	8
Methoxy-	-H	25	33	42
Ethoxy-	-H	12	10	10
Propoxy-	-H	1	3	10
Isopropoxy-	-H	5	5	24

* Control seed soaked in water alone averaged 5 to 8 % germination.

under identical conditions, followed by germination in the light, yielded values comparable to the water controls.

In addition to the above mentioned compounds, some additional purines were studied, and those compounds which possessed stimulation of seed germination activity in the dark included the 6-(*o*-, *m*-, and *p*-)chlorophenyl)aminopurines, as well as 6-*N*-methylphenyl- and 6-(3-isopropoxypropyl)aminopurines (table II). Other substituted groups (i.e., hydrazino- and chloro-) on the 6 position of the purine ring did not yield a very active compound; however, some significant activity was observed when a methoxy group was attached at the 6 position. The higher homologues in the hypoxanthine series were considerably less active, and the corresponding methylamino- and methylthiopurine analogues were inactive (3). Further, when the 6-methoxypurine structure was modified by the addition of a methyl substituent in the imidazole portion of the nucleus, this disubstituted purine derivative was then found to be inactive in the assay system.

It appears, that while the substituted group in the 6 position of the purine nucleus may vary within wide limits, perhaps the purine moiety itself is more specific for seed germination activity. It is interesting to observe that the appropriate group attach at the 6 position in the purine nucleus may be connected with either nitrogen, sulfur or oxygen and still produce an anologue which retains biological activity.

The authors wish to acknowledge the technical asistance of Mr. D. R. Tuttle.

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